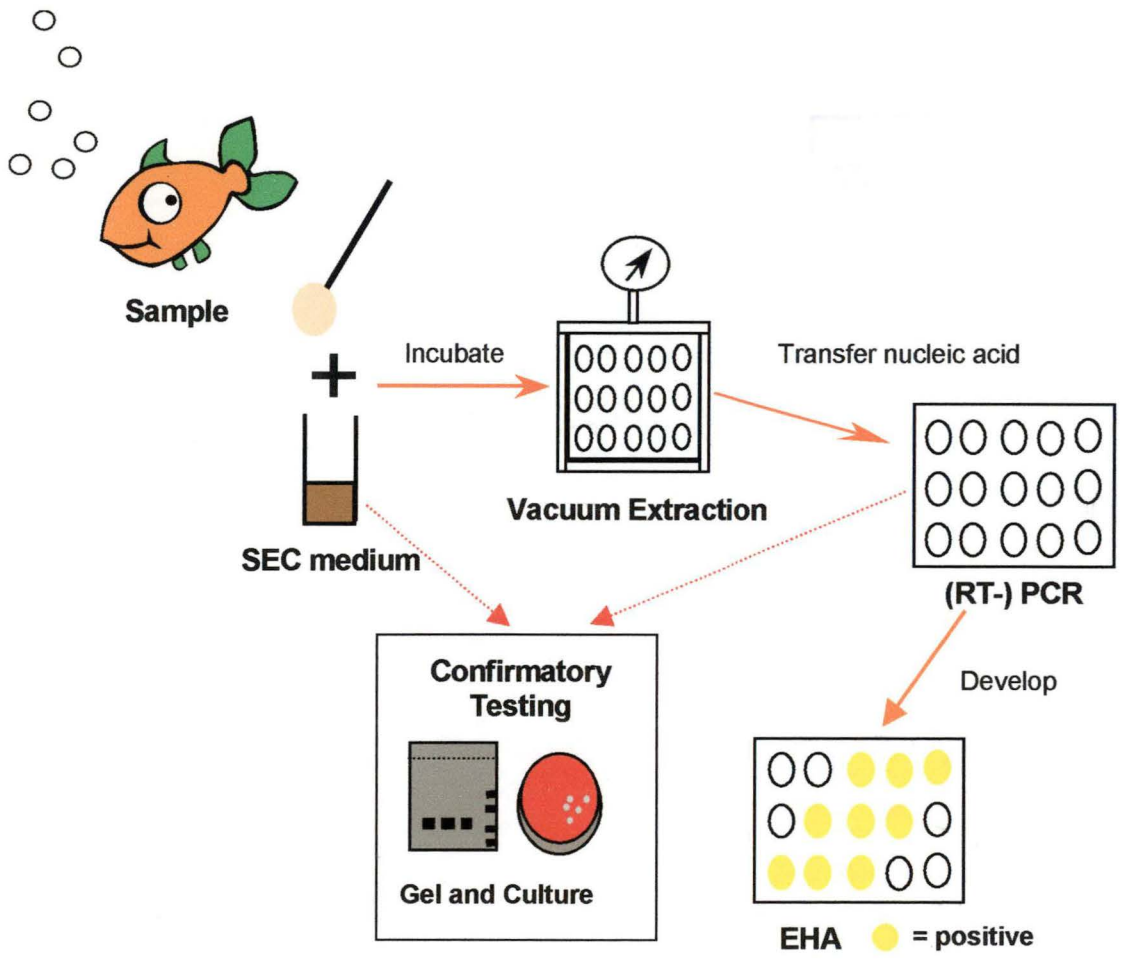
Development of a Streamlined, Selective-Enrichment Culture, One-Tube (RT-)PCR-Enzyme Hybridization Assay To Detect Bacterial Fish Pathogens in Covertly Infected Farmed Salmonids



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Submitted in fulfilment of the requirements for the

Degree of Doctor of Philosophy

University of Tasmania (November, 2003)

Declaration

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Teresa Kaye Wilson

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Abstract

A new system which offers a low-cost and high performance means for detecting four bacterial pathogens *Aeromonas salmonicida*, *Tenacibaculum maritimum*, *Lactococcus garvieae* and *Yersinia ruckeri* in covertly infected farmed salmonid fish has been developed. The system couples Selective Enrichment Culture (SEC), Polymerase Chain Reaction (PCR) and Enzyme Hybridization Assay (EHA) technologies to provide streamlined high-throughput sample processing suitable for large scale surveillance and monitoring programs.

The SEC media used for the technology were those developed by T.Wilson and J.Carson for the Cooperative Research Centre (CRC) for Aquaculture Ltd, except for the *A. salmonicida* medium which was developed using information obtained during the CRC project and by performing Minimum Inhibitory Concentration (MIC) assays with 32 antimicrobial agents that were not previously tested. Laboratory sensitivity of the *A. salmonicida* medium was 100% as determined by Most Probable Number (MPN) analysis and specificity of the medium was 97%.

Bacterial DNA and RNA were extracted from the SEC media using guanidinium isothiocyanate (GuSCN) and Whatman Polyfiltronics GF/B 96-well Uni-filter plates. Sensitivity of the system as determined by PCR was between 1 and 16 CFU per 200 µl of selective-enrichment medium for DNA and between 1 and 9 CFU per 200 µl of selective-enrichment medium for RNA. The use of vacuum filtration and the 96-well glass microfibre filter plate allowed for rapid high-throughput and inexpensive extraction.

Due to the high sensitivity of PCR, the technology is prone to false-positive results due to amplicon carry-over. To help prevent the occurrence of these erroneous results the photochemical IP-10 was added to the procedure. IP-10 inactivates PCR amplicons rendering them incapable of re-amplification in subsequent PCR reactions.

NucleoLink™ PCR-enzyme hybridization strips were used to perform sensitive streamlined (RT)PCR-EHA. For each bacterium 4 fg of pure target DNA or RNA was detected. With this system only one tube per sample from

cDNA to EHA was required, decreasing the cost and time involved in sample transfer and decreasing the risk of cross-contamination between samples.

In laboratory trials the final SEC-(RT-)PCR-EHA system proved to be very sensitive with 1-16 CFU from selective-enrichment culture detected. Specificity of the system was >99%. The system was also rapid with the 96-well nucleic acid extraction to EHA results achieved in as little as 8 hours.

Test validation with field samples was achieved by determining test specificity and sensitivity from an epidemiological perspective. During this testing the sensitivity and specificity of the system matched that obtained using purified nucleic acids in the laboratory. Validation was conducted using a total of 10 fish trials using fish from different environments and from different sample sources.

The system described here uses two types of technology, PCR and RT-PCR. The SEC-PCR-EHA system detects genomic DNA from the target pathogen, demonstrating evidence of infection, past or present and is ideal for use in surveillance programs and for quarantine. The SEC-RT-PCR-EHA system detects ribosomal RNA from the target pathogens. This system gives a more accurate indication of the presence of live bacteria and therefore of live covert infection and is useful when monitoring changes in the disease status of a population of fish over a short period of time. The system was developed using inexpensive materials instead of proprietary products, and disposable equipment usage was minimised in an effort to keep the system low-cost. The sensitivity of the system was maximised to ensure the detection of covertly infected fish and specificity of the system was as good as the PCR primers would allow. The system utilises 96-well technology to minimise the volume of reagents and to enable streamlined sample processing using a multichannel pipette. In summary, a low-cost, high-performance and streamlined high-throughput sampling system has been developed.

Acknowledgments

This research has been made possible by funding obtained from the Fisheries Research and Development Corporation (FRDC), and the use of the facilities at the Tasmanian Department of Primary Industries Water and Environment (DPIWE).

The research has benefited significantly from help and support of many people. Firstly I would like to thank Dr. Jeremy Carson, DPIWE for his assistance and guidance during the research and the preparation of the thesis. I would also like to thank Dr. John Bowman, University of Tasmania for his assistance throughout this time.

I wish to thank Jane Oakey from the University of Technology, Sydney, and Annette Thomas from the Queensland Department of Primary Industries, Australia, for their kind donation of DNA for use in specificity testing of the PCR. Many thanks go to Nick Gudkovs from the Australian Animal Health Laboratory, Geelong, for sequencing the *Aeromonas hydrophila* that was isolated during one of the fish trials.

I wish to thank John Tessman from Cerus Corporation, Concord, California, for the supply of IP-10. Gary Fahle, National Institutes of Health, Bethesda, Maryland, and David Persing Mayo Clinic, Rochester, Minnesota, for their advice on the use of the chemical.

Many thanks go to: Kevin Chilman, Sevrup Pty Ltd; Harry King, Saltas Pty Ltd; Ron Morrision, Southern Ocean Trout; and Jo Sadler, Nortas Pty Ltd, for the supply of fish for field testing. Thank you to Kevin Ellard, Fish Health Unit, DPIWE for helping with field trial sample collection.

Thank you to Duncan Clark, GeneSys Ltd, Farnborough, UK, at the sci.bionet.methds-reagents newsgroup for some wonderful advice on DNA extraction, maximising PCR template volume and the use of DNase cofactors

for RT-PCR. Also many thanks go to Chris Baldock, AusVet Animal Health Services, Brisbane for valuable advice regarding test validation in the field.

I wish to thank all of my colleagues at the DPIWE, especially Toni Wagner, Dr. Mark Lleonart and Dr. Marianne Douglas-Helders for their support throughout the project and for making my working environment enjoyable.

Last but not least I would like to thank my family for their love and support and especially Chris for giving me the time, space and encouragement to finish this thesis and for being an excellent proof reader – and for looking after Dominic! Oh and thank you Dominic for… um.. for your hugs.

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List of Abbreviations

1-MeIm	1-methylimidazole
ATCC	American Type Culture Collection
AU$	Australian dollars
bp	base pair
BCIP	5-Bromo-4-chloro-3-indolyl-phosphate
BSA	bovine serum albumin
CDS	calibrated dichotomous sensitivity (test)
CFU	colony forming unit
CORT	selective enrichment medium for *Lactococcus garvieae*
CRC	Cooperative Research Center
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DNase	deoxynuclease
dNTPs	deoxynucleotide triphosphates
DIAPOPS	Detection of Immobilised Amplified Product in a One-Phase System
DPIWE	Department of Primary Industries Water and Environment
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EDTA	ethylene-diamine-tetra acetic acid
EF	enrichment factor
EHA	enzyme hybridization assay
EI	enrichment index
ELISA	enzyme linked immuno sorbent assay
ER	enrichment ratio
FAT	fluorescent antibody test
fg	femtogram
g	gravities
GF/B	glass fibre Uni-filter plate (Whatman)
GuSCN	guanidinium isothiocyanate
HK3C	selective enrichment medium for *Aeromonas salmonicida*
IP-10	isopsoralen compound 10
IR	initial ratio

KPa	kilopascals
L6	lysis buffer 1
L2	lysis buffer 2
MIC	minimum inhibitory concentration
mJ	millijoules
mM	millimole
MPN	most probable number
nm	nanometre
NBT	nitro blue tetrazolium
NCCLS	National Committee for Clinical Laboratory Standards
ng	nanogram
OD	optical density
PAAS	plasmid based PCR assay for *Aeromonas salmonicida*
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pg	picogram
PLC	productivity limiting concentration
PNPP	*p*-nitrophenylphosphate
POSI	selective enrichment medium for *Tenacibaculum maritimum*
POST	selective enrichment medium for *Yersinia ruckeri*
RT-PCR	reverse transcriptase polymerase chain reaction
RNA	ribonucleic acid
RNase	ribonuclease
RO	reverse osmosis (water)
SAP	streptavidin alkaline phosphatase
SBA	sheep's blood agar
SDS	sodium dodecyl sulphate
SE	standard error
SEC	selective enrichment culture
sp.	species
spp.	more than one species
ssp.	subspecies
SSC	salt sodium citrate
TAE	Tris-acetate-EDTA

Taq	*Thermus aquaticus*
TE	Tris-EDTA
T_m	melting temperature of DNA
Triton X 100	t-Octylphenoxypolyethoxyethanol
UV	ultra violet (light)
*vap*A	PCR assay for *Aeromonas salmonicida* which targets the *vap*A gene
v/v	volume per volume
w/v	weight per volume

CHAPTER 1: GENERAL INTRODUCTION

1.1. Australian bacterial disease agents of farmed salmonid fish

Fish farming, like the farming of any animal, places the fish in an unnatural and often compromised environment. Areas of possible compromise include water quality, nutrition, stocking density and social hierarchy pressures (Schreck 1981). These compromises inevitably lead to stress which in turn leads to a reduction of immune competence (Langdon 1988) which leaves the fish vulnerable to disease agents.

Fortunately Australia has few serious fish disease agents compared with most other countries. The lack of the disease agents such as the furunculosis ('typical') strain of *Aeromonas salmonicida*, enteric red mouth *Yersinia ruckeri* (serotype O1a) and *Renibacterium salmoninarum* allow for low antibiotic use and the production of high quality fish. The Australian fin-fish industry is small compared with most other countries though each year the industry becomes a more substantial part of the Australian economy. In the 2000/2001 financial year fin-fish aquaculture was estimated to be worth AU$376 million to the Australian economy, a 17.5% increase on the previous financial year (O'Sullivan and Dobson 2003). The relative small size of the industry means that any market advantage that exists due to a lack of disease must be vigorously protected to ensure the survival of the industry. The Australian fin-fish farming is not completely free of bacterial diseases however, the major disease agents found in temperate Australian fin-fish are given in Table 1.1. While all of these bacteria are important pathogens to Australian fin-fish farmers, four species have been identified as having a high priority for diagnosis, monitoring and control for the Tasmanian salmonid industry (Carson 1998). These bacteria are *A. salmonicida*, *Tenacibaculum maritimum* (formerly *Flexibacter maritimus*), *Lactococcus garvieae* and *Y. ruckeri.*

An important characteristic of these pathogens is their capability of residing in fish in very low numbers, establishing a carrier status. In this form of covert infection, fish show no signs of disease but the bacterium is spread naturally in water from infected to uninfected fish (McCarthy 1983), and it is only when the fish

are stressed that the disease becomes evident. Therefore, under aquaculture conditions, by the time the disease becomes evident a large proportion of stock may be infected. Consequently, the detection of asymptomatic carriers is an essential strategy for effective disease control (Bullock and Stuckey 1975).

Table 1.1: Significant bacterial pathogens of temperate farmed fin fish in Australia (adapted from Carson 1998)

Pathogen	Disease	Host range
Aeromonas salmonicida (atypical)	Goldfish ulcer disease	goldfish silver perch salmonids
A. salmonicida ssp *lerunnica*	Flounder ulcer disease	greenback flounder striped trumpeter salmonids
A. salmonicida bv acheron	Marine aeromonad disease	Atlantic salmon
Carnobacterium piscicola	Egg peritonitis	salmonids
Edwardsiella tarda	Edwardsiellosis	rainbow trout
Flavobacterium psychrophilum	Fin erosion	Atlantic salmon
Lactococcus garvieae	Streptococcaceosis	rainbow trout
Tenacibaculum maritimum	Cutaneous erosion disease	salmonids marine fish
Vagococcus salmoninarum	Egg peritonitis	salmonids
Vibrio anguillarum	Vibriosis	salmonids marine fish
Yersinia ruckeri (O1b)	Yersiniosis	salmonids

1.2. The four target bacterial fish pathogens

Aeromonas salmonicida.

The bacterium *A. salmonicida* is separated into 5 subspecies: *A. salmonicida* ssp. *salmonicida*, *A. salmonicida* ssp. *achromogenes*, *A. salmonicida* ssp. *masoucida*, *A. salmonicida* ssp. *smithia* (Holt, *et al.* 1994) and *A. salmonicida* ssp. *pectinolytica* (Pavan, *et al.* 2000). In addition to the recognised subspecies there is a heterogenous collection of strains, which have the essential characteristics of

the species, but have phenotypic characters unlike those of the named subspecies (Austin and Austin 1993). These strains are referred to as "atypical".

Globally, *A. salmonicida* is recognised as a major pathogen causing disease in farmed and wild fish. *A. salmonicida* is the causative agent of the disease furunculosis. Disease caused by *A. salmonicida* may seriously compromise production of cultured fish through direct losses, cost of treatment and control, market rejection and restrictions on movements of stocks. For example, in the late 1980s, the Norwegian salmon aquaculture industry crashed because of an outbreak of furunculosis transmitted in apparently healthy smolts imported from Scotland (Bernoth and Worland 1995/96).

Fish furunculosis was first reported in hatchery trout from Germany by Emmerich and Weibel (1894); the bacterium was referred to as *Bacterium salmonicida*. Since then, the disease has been found in most countries and is the cause of significant losses throughout Europe and the United States. Furunculosis in salmonids may appear in three forms: chronic, acute and peracute. Chronic disease occurs mainly in older fish and may be accompanied by the development of red, raised, fluid-filled lesions called 'furuncules'. Acute disease occurs in younger fish and appears as a generalised septicaemia followed by high mortality levels. Peracute disease only occurs in juvenile fish and is characterised by its sudden onset, absence of any specific signs of disease and very high losses of fish (Austin and Austin 1993).

Furunculosis in salmonids is most often caused by "typical" *A. salmonicida* defined as *A. salmonicida* ssp. *salmonicida*, and has not been reported in Australia. However, in Europe and America "atypical" strains also cause disease, particularly in sea-caged salmonids (Austin and Austin 1993). Importantly from a local perspective, three "atypical" strains of *A. salmonicida* have been identified in Australia and are known to cause disease in several species of fish.

The most well known of the three Australian *A. salmonicida* strains causes goldfish ulcer disease (GUD), and is thought to have been imported from Japan with goldfish (*Carassius auratus*) broodstock in 1974 (Humphrey and Ashburner 1993). The disease in Australia was diagnosed in July 1977 from ulcerated fish

from a farm in Gippsland, Victoria (Trust, *et al.* 1980). Since its recognition, GUD has continued to spread in aquarium stocks of goldfish distributed throughout Australia and has probably spread to wild stocks of fish through the practice of anglers using live goldfish as bait (Whittington and Cullis 1988) and the disposal of unwanted goldfish into waterways. The disease is now considered enzootic in south-eastern Australia, excluding Tasmania.

The second strain of *A. salmonicida* causes flounder ulcer disease (FUD) and was recovered from ulcerative dermal lesions and kidneys of marine greenback flounder (*Rhombosolea tapirina*) in Tasmania (J. Carson unpubl. data). This strain is phenotypically different from other "atypical" strains of *A. salmonicida* and is a fastidious organism that only grows slowly in culture, forming very small colonies.

Whittington and Cullis (1988) showed the potential of *A. salmonicida* to cause disease in the Australian salmonid industry by experimentally demonstrating that Atlantic salmon (*Salmo salar* L.), brown trout (*S. trutta*), brook trout (*Salvelinus fontinalis*) and rainbow trout (*Oncorhynchus mykiss*) are susceptible to the GUD strain. However, it was over 10 years later that an atypical strain of *A. salmonicida* was isolated as the causative agent of an actual disease outbreak in Australian farmed salmonids. In 1999 *A. salmonicida* was recovered from moribund Atlantic salmon growing in estuarine conditions in Macquarie Harbour, Tasmania. The strain was "atypical" but phenotypically different to either the FUD or GUD strain of the bacterium. Because of its recent discovery little is known about the virulence of the bacterium or its natural reservoir. The bacterium has been given the provisional strain designation of *A. salmonicida* biovar acheron (J. Carson unpubl. data).

Lactococcus garvieae.

L. garvieae is the causative agent of disease in a wide variety of aquatic species and environments from rainbow trout grown in temperate freshwater (Carson and Munday 1990) and tropical giant freshwater prawns (*Macrobranchium rosenbergii*) (Chen, *et al.* 2001), to yellowtail (*Seriola quinqueradiata*) cultured in the marine environment. In Australia *L. garvieae* causes a septicaemic disease in

rainbow trout called streptococcaceosis (Carson, *et al.* 1993). Fish death due to *L. garvieae* is associated with poor water quality and, with the increase of fish farming worldwide, it has become a major problem (Zlotkin, *et al.* 1998a). Fortunately, due to improved husbandry techniques in Australia, disease caused by *L. garvieae* has been in decline in the last few years. However, the organism is still a threat with the last reported outbreak occurring in rainbow trout from NSW in 1999.

Yersinia ruckeri.

Y. ruckeri was first reported as a fish pathogen in the Hagerman valley in Idaho in the 1950's (McDaniel 1971). The causative agent of this outbreak is now referred to as the "Hagerman" strain, a strain of the bacterium that causes the disease known as Enteric Red Mouth (ERM) in rainbow trout. ERM is an acute disease that in classic disease outbreaks causes 'redmouth' symptoms and high mortality rates. However, *Y. ruckeri* is capable of causing acute and chronic disease in a wide variety of fish species. The severity of the disease depends on the biotype of *Y. ruckeri* and the host.

For identification purposes *Y. ruckeri* can be grouped according to serotype. In this scheme there are four major serotypes 01, 02, 03, and 04 (Romalde, *et al.* 1993). The serotype 01 has 2 subgroups, 01a and 01b, and in serotype 02 3 subgroups, 02a, 02b and 02c, have been recognised. Most *Y. ruckeri* strains belong to serotype 01 and from this group 6 clonal types can be recognised, the most virulent of which are types 2 and 5. All Australian strains have been placed in the less-virulent types 1 and 3. In Australia disease caused by *Y. ruckeri* occurs predominantly in Atlantic salmon and in all tested cases the causative agent has belonged to serotype 01b. This disease is referred to as Yersiniosis (Llewellyn 1980). Yersiniosis is usually chronic and associated with stress due to poor water quality or elevated temperature.

Tenacibaculum maritimum.

T. maritimum (formerly *Flexibacter maritimus*) is part of a group of bacteria classified as *Cytophaga-Flavobacterium-Bacteroides* (CFB) which constitute one of the dominant bacterial groups in the marine environment (Bowman, *et al.* 1997). *T. maritimum* was first reported as a fish pathogen of sea bream farmed in Japan in the 1970s. It was classified as *F. maritimus* by Wakabayashi, *et al.* (1986) and later reclassified as *T. maritimum* by Suzuki, *et al.* (2001). The disease is associated with handling stress and can, if untreated, lead to significant losses. The pathogen has world-wide distribution (Bernardet 1997) and has a wide host range, being identified as a cause of skin erosions in many commercially farmed fish species. In Australia, farmed or experimentally held fish susceptible to *T. maritimum* include Atlantic salmon, rainbow trout, greenback flounder, and striped trumpeter (*Latris lineata*).

1.3. Conventional methods for the detection of fish disease

The detection of bacterial diseases in fish mostly relies on the recovery of the causative organism in culture, and the analysis of phenotypic or serological properties of the pathogen. For culture, samples are usually taken from fish that have recently died or that appear unwell. In this case a large number of the pathogen are usually present, and identification is typically straight-forward. However, this method is not efficient when only low numbers of the target bacterium are present as the sample taken for culture is small and the target bacterium is likely to be missed. Also, the target bacterium may be non-culturable or normal fish flora may be present in higher numbers than the target, making identification of any target pathogen that is present difficult.

Fluorescent antibody test (FAT) is often used for the identification of fish pathogens. However, like culture, the sensitivity of the test is poor (Armstrong, *et al.* 1989) with a positive result requiring the pathogen to be present in sufficient numbers to be detected by microscopy. Typically, this requires about $1x10^6$ bacteria g^{-1}, although with sample concentration and examination of 100 fields of view, it is possible to detect as little as $1x10^3$ cells g^{-1} of *Renibacterium*

salmoninarum (Sakai, *et al.* 1989). The specificity of FAT is usually high, particularly if a monoclonal antibody is used. However, due to the commonality of antigens amongst related groups of bacteria the results are not always conclusive. Another form of immunoassay sometimes used in the detection of fish disease is immunoblotting or dot-blotting. Cipriano *et al.* (1985) used a dot-blot test to detect *Vibrio anguillarum* and the test was found to be as specific as FAT but about 100 times more sensitive.

It is clear then that conventional methods work well for the detection of fish disease but lack the sensitivity required to detect pathogens that are capable of residing in the fish in very low numbers. In order to control the spread of disease in a fish population detection of covertly infected fish is very important. The standard procedure for detecting covert infections in fish is to perform a 'stress test' on a sample-set from the population. This procedure was originally developed for detection of carriers of *A. salmonicida* by Bullock and Stuckey (1975). In such procedures fish are treated with an immunosuppressor such as prednisolone and held at an elevated temperature, usually 18°C. This treatment suppresses immune function therefore providing an opportunity for expression of the pathogen's virulence factors, resulting in bacterial amplification and development of a fulminating disease. Heat and corticosteroid stress testing is considered to be the most reliable method for detecting asymptomatic carriage of *A. salmonicida* (Hiney, *et al.* 1994). However the procedure may take up to three weeks to complete, requires specialised facilities and is resource intensive.

While all of the above methods have their place in the diagnosis of fish diseases, due to the lack of sensitivity or time involved in processing the samples none are suitable for the high-throughput detection of covert infections.

1.4. Methods suitable for high-throughput detection of bacterial infections

Bacterial detection methods that are suitable for high-throughput sampling are generally restricted to those that are able to be miniaturised. Suitable tests include:

the enzyme linked immunosorbent assay (ELISA); restriction enzyme digestion; probe hybridization and polymerase chain reaction (PCR).

Enzyme Linked Immunosorbent Assay (ELISA).

An ELISA test usually has better sensitivity that other immunological methods making it more suitable for the detection of covert pathogens. This increase in sensitivity occurs mostly as a result of sample concentration when the antigen is captured by an immobilised capture antibody in a microtitre tray, thus making the test suitable for high-throughput sampling. Capture ELISA has been used to detect several bacterial fish pathogens including *A. salmonicida* (Bernoth 1990), *Vibrio anguillarum* (Romestand, *et al.* 1993), and *R. salmoninarum* (Gudmundsdottir, *et al.* 1993). Despite the ability of capture ELISA to concentrate bacteria, the sensitivity of the system is typically no better than $1x10^5$ cells (Swaminathan and Feng 1994), although Hiney *et al.* (1994) were able to detect as little as $1x10^4$ CFU ml^{-1} of *A. salmonicida*.

Probe hybridization.

A nucleic acid probe is a short length of single stranded DNA (oligonucleotide probe) of known sequence of nucleic acids that are characteristic of the target bacterium. Using the appropriate hybridization conditions such as temperature and probe concentration, the probe binds only to the complimentary nucleic acid sequence and not non-specifically to other areas of the genome (Cunningham 2002). Nucleic acid probes are labelled with a radioactive marker or a chemical tag such as biotin or digoxigenin so that they can be detected by antibody binding coupled to fluorescent, chemiluminescent or colorimetric detection techniques (Nagata, *et al.* 1985; Gabrielle, *et al.* 1993; Tyagi and Kramer 1996; Bains 1998; Gutierrez, *et al.* 1998). For ease of detection the hybridized molecule is usually captured on a solid support such as microscope slides, nylon, nitrocellulose, latex or magnetic beads, or in microtitre plates. Probe hybridization has been used to detect the presence of many bacterial fish pathogens. Moreno, *et al.* (1999) used *in situ* slide examination and dot-blot to determine whether eel slime was a

potential reservoir for fish pathogens, *Proteobacteria* such as vibrios and aeromonads were found to be abundant. Dot-blot hybridization has also been used to detect *V. anguillarum* (Martinez-Picado, *et al.* 1994; Powell and Loutit 1994) and *R. salmoninarum* (Hariharan, *et al.* 1995). Colony blot hybridization is a rapid method for identifying bacterial colonies growing on an agar plate. The method has been used to identify *V. anguillarum* and *V. ordalii* (Ito, *et al.* 1995) and *V. scopthalmi* (Cerda-Cuellar and Blanch 2002). Therefore while nucleic acid probes are useful for the diagnosis of fish pathogens that are present in numbers sufficient to be found *in situ* or by blotting methods, for applications where a high sensitivity is required gene probes can be used as primer pairs in the polymerase chain reaction (PCR).

Polymerase Chain Reaction (PCR).

PCR is a well-documented and accepted tool for the detection of fish pathogens (León, *et al.* 1994b; Magnússon, *et al.* 1994, Høie, *et al.* 1996, Carson 1998). PCR is useful for the detection of microorganisms for which culture and serological methods are difficult, time consuming, extremely expensive or lack sensitivity or specificity. PCR involves selective amplification of a unique segment of DNA, amplification will only occur if a pair of specific oligonucleotides have hybridized to the target sequence of DNA. Successful amplification of DNA can lead to the formation of approximately a million copies of the target sequence within 2 or 3 hours of cycling (Saiki, *et al.* 1988), see Figure 1.1. After PCR the detection of the amplified sequence is achieved by electrophoresis where DNA fragments of different sizes are separated in a gel substrate. Visualization of the DNA is achieved by staining with ethidium bromide and viewing under UV light.

Because of the speed and sensitivity of PCR (Pascho, *et al.* 1998) the last seven years have seen a significant increase in the design of new PCR primers for fish pathogens, and the use of PCR as a diagnostic tool in laboratories. From Table 1.2 it can be seen that many researchers have chosen to develop PCR based on the 16S small-subunit ribosomal ribonucleic acid (rRNA) region of the genome. The 16S region is often chosen for PCR primers as it is highly conserved (Pace 1973),

and therefore stable and predictive. While this region is conserved, within the region there are hypervariable areas that contain characteristic signatures that often define a species (Stackebrandt and Goebel 1994). A disadvantage of using the 16S region of the genome for PCR is that the region may lack definitive information, which is required for probe specificity. The design of a specific probe normally leads to a high predictability of species identification.

When there is insufficient information in the 16S rRNA gene to differentiate species, it may be necessary to design a probe to target another area of the genome. Recently many researchers have targeted the 16S-23S rDNA intergenic spacer region of the genome in order to develop species specific PCR, see Table 1.2, or to investigate molecular differences within the same species (Trevisanato, *et al.* 1996; Cunningham, *et al.* 1997; Grayson, *et al.* 1999). The 16S-23S rDNA intergenic spacer region has proven useful for closely related species as it varies more significantly in size and sequence among closely related bacterial species than the 16S or 23S rRNA genes. While many species specific PCR assays have been developed using the 16S-23S rDNA intergenic spacer region, care must be taken when developing new probes that the bacterium has only 1 rRNA operon (Frothingham and Wilson 1993). A few researchers have targeted other areas of the genome, Aoki, *et al.* (2000) found specificity problems with 16S rDNA *L. garvieae* PCR but found the dihydropteroate synthase gene to be highly specific for the bacterium. A long term problem for fish farmers and researchers has been the identification of the bacterium *A. salmonicida* as 16S rDNA primer sets are not species specific and many attempts have been made to produce a more specific and all-encompassing alternative. Plasmid-bourne PCR primers, PAAS PCR, were developed for *A. salmonicida* (Hiney, *et al.* 1992) that are very specific with no cross-reaction with DNA from other species documented. However, not every *A. salmonicida* strain contains the target plasmid, allowing for the possibility of false-negative reactions (Byers, *et al.* 2002). A further PCR for *A. salmonicida* was developed to target the *vap*A gene which codes for the paracrystalline surface protein layer (A-layer) associated with virulence in *A. salmonicida* (Chu, *et al.* 1991). However Byers *et al.* (2002) demonstrated that this PCR did not detect

every strain of *A. salmonicida* either. To date there is no all-encompassing, species specific PCR for *A. salmonicida*.

Recently a quantitative form of PCR, real-time PCR has been used for the detection and quantification of nucleic acids. Real-time PCR is comparable to conventional PCR in terms of sensitivity and specificity however, results are achieved in less time (usually around 30 minutes), are quantitative and are immediately available for computer based analysis (Ke, *et al.* 2000). Real-time PCR has been used to detect infectious haematopoietic necrosis virus in salmonids (LaPatra, *et al.* 2001; Overturf, *et al.* 2001), red seabream iridovirus (Caipang, *et al.* 2003) and Edwardsiella ictaluri in channel catfish (Bilodeau, *et al.* 2003). A significant draw back of this technology is the cost of purchasing a real-time PCR thermal cycler, at around $100 000 ($Au) this equipment will remain out of the reach of most small veterinary laboratory for some time.

While PCR has been successfully applied to the detection of many fish pathogens, due to limitations such as sample size, the presence of non-target nucleic acids and PCR inhibitors the sensitivity of the technique with field samples has not been as high as expected. This is demonstrated in the use of nested reverse transcriptase PCR to detect *R. salmoninarum* from ovarian fluid and kidney reported by Magnússon *et al.* (1994). In this report efficacy of PCR was compared with both culture and ELISA. While PCR was more sensitive than culture or ELISA, not every ELISA positive or culture positive sample was detected by PCR. Also, the authors found that the PCR enzyme *Taq* polmerase was inhibited by kidney tissue. Khan and Cerniglia (1997) developed a 16S rRNA gene primer set to detect *Aeromonas caviae* and *Aeromonas trota* by PCR. The limit of detection of the PCR with purified genomic DNA was 0.1 ng however, the limit of detection in crab meat was only 50-100 cells g^{-1}. Gustafson *et al.* (1992) detected 10^4 CFU g^{-1} of *A. salmonicida* from seeded kidney, faeces and spleen samples and the authors found that this detection limit could be lowered if a pre-enrichment step was added to the protocol. Other detection limits for *A. salmonicida* from fish tissue include 200 genome equivalents g^{-1} of sample (O'Brien, *et al.* 1994) and 10^4-10^5 CFU ml^{-1} (Høie, *et al.* 1997; Byers, *et al.* 2002). With this level of detection PCR is not

significantly more sensitive than culture (as long as the cells are culturable) or immunological methods for detecting bacteria from field samples. This level of detection requires the fish to be carrying the pathogen in numbers in excess of the level expected in a covert infection (Crane and Bernoth 1996). Clearly for the detection of covertly infected fish the limitations of PCR must be overcome.

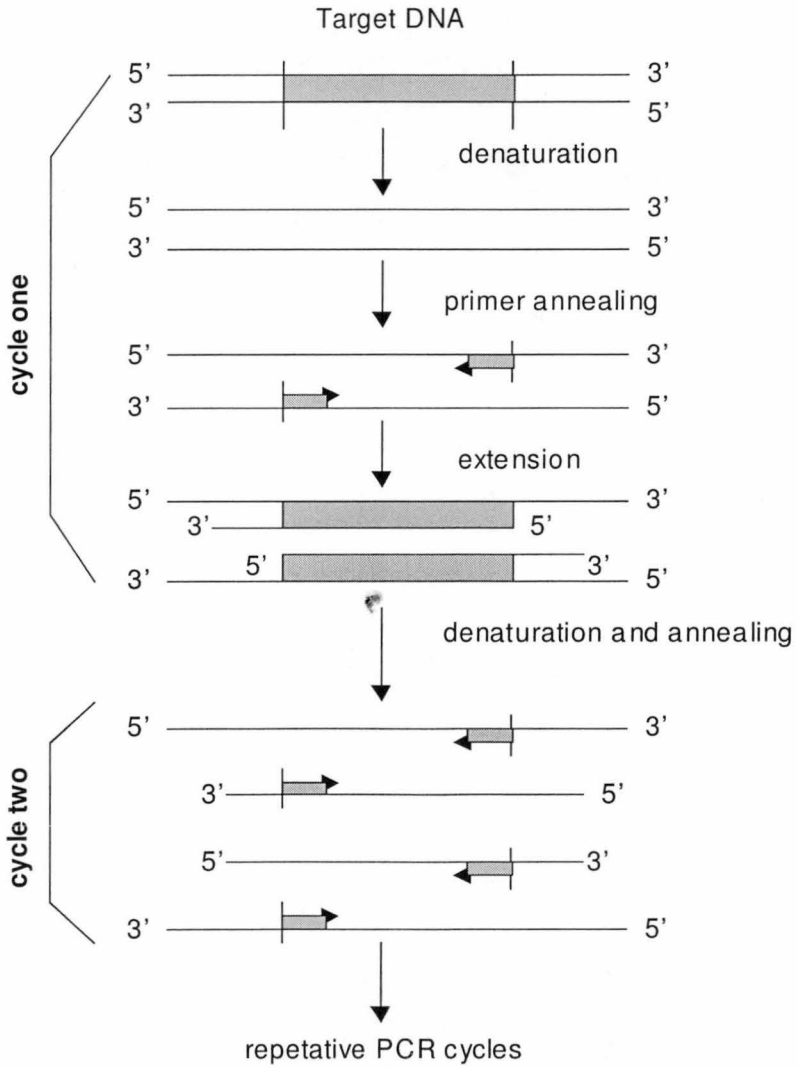


Figure 1.1: Diagram of PCR showing the amplification of a target region (Atlas and Bej 1994).

Table 1.2: Gene probes for PCR for the detection of bacterial fish pathogens.

Pathogen	Target	Reference
Aeromonas caviae	16S rRNA gene	Khan and Cerniglia 1997
Aeromonas trota	16S rRNA gene	Khan and Cerniglia 1997
Aeromonas salmonicida	16S rRNA gene	Høie, *et al.* 1996
	16S rRNA gene	Carson 1998
	PAAS	Hiney, *et al.* 1992
	vapA gene	Gustafson, *et al.* 1992
	Asal-3 clone	Miyata, *et al.* 1996
Aeromonas hydrophila	16S rRNA gene	Dorsch, *et al.* 1994
	lip gene	Cascón, *et al.* 1996
Aeromonas schubertii	16S rRNA gene	Ash, *et al.* 1993
Flavobacterium columnare	16S rRNA gene	Bader and Shotts 1998
	16S rRNA gene	Bader, *et al.* 2003
Flavobacterium	16S rRNA gene	Toyama, *et al.* 1994
psychrophilum	16S rRNA gene	Bader and Shotts 1998
	16S-23S intergenic spacer rRNA	Ballarda, *et al.* 2002
Lactococcus garvieae	16S rRNA gene	Carson 1998
	Dihydropteroate synthase gene	Aoki, *et al.* 2000
	16S rRNA gene	Zlotkin, *et al.* 1998a
Mycobacterium marinum	16S rRNA gene	Knibb, *et al.* 1993
Photobacterium damselae ssp. *piscicida*	16S rRNA gene	Osorio, *et al.* 1999
Photobacterium damselae	16S rRNA gene	Osorio, *et al.* 1999
ssp. *damselae*	ureC gene	Osorio, *et al.* 2000
Renibacterium	16S rRNA gene	Magnússon, *et al.* 1994
salmoninarum	p57 gene	Brown, *et al.* 1994
	pMAM29 clone	León, *et al.* 1994a

Table 1.2 continued:

Pathogen	Target	Reference
Streptococcus agalactiae	16S-23S intergenic spacer rRNA	Berridge, *et al.* 2001
Streptococcus difficilis	16S-23S intergenic spacer rRNA	Berridge, *et al.* 2001
Streptococcus iniae	16S rRNA gene	Zlotkin, *et al.* 1998b
	16S-23S intergenic spacer rRNA	Berridge, *et al.* 1998
Tenicibaculum maritimum	16S rRNA gene	Bader and Shotts 1998
	16S rRNA gene	Carson 1998
Vagococcus spp.	16S rRNA gene	Williams, *et al.* 1992
Vibrio cholerae	16S-23S intergenic spacer rRNA	Chun, *et al.* 1999
Vibrio fluvialis	16S-23S intergenic spacer rRNA	Lee, *et al.* 2002
Vibrio mimicus	16S-23S intergenic spacer rRNA	Chun, *et al.* 1999
Vibrio nigripulchritudo	16S-23S intergenic spacer rRNA	Lee, *et al.* 2002
Vibrio parahaemolyticus	16S-23S intergenic spacer rRNA	Maeda, *et al.* 2000
Vibrio penaeicida	16S rRNA gene	Genmoto, *et al.* 1996
Vibrio proteolyticus	16S-23S intergenic spacer rRNA	Lee, *et al.* 2002
Vibrio salmonicida	16S-23S intergenic spacer rRNA	Lee, *et al.* 2002
Vibrio splendidus	16S-23S intergenic spacer rRNA	Lee, *et al.* 2002
Vibrio trachuri (*Vibrio harveyi*, Thompson, *et al.* 2002)	PST-I clone	Iwamoto, *et al.* 1995
Vibrio harveyi	ToxR gene	Conejero and Hedreyda 2003
Vibrio tubiashii	16S-23S intergenic spacer rRNA	Lee, *et al.* 2002
Vibrio vulnificus	23S rRNA gene	Arias, *et al.* 1995
	haemolysin gene	Coleman, *et al.* 1996
Yersinia ruckeri	A fragment	Argenton, *et al.* 1996
	16S rRNA gene	Carson 1998
	16S rRNA gene	Gibello, *et al.* 1999
	16S rRNA gene	LeJevne and Rurangirwal 2000

1.5. Coupling ELISA and PCR with selective-enrichment culture to detect covert infections

Despite the sensitivity currently achievable with ELISA and PCR, the presence of non-target cells and the restriction of small sample size mean that low-level or covert infections may be missed. Adding an enrichment step to the procedure can significantly increase the likelihood of detecting covert infections. Selective-enrichment increases the amount of target bacterium and helps reduce the number of competing microflora (Fitter, *et al.* 1992; Swaminathan and Feng 1994; Witham, *et al.* 1996). Selective-enrichment has been coupled with ELISA and PCR in the food industry to improve sensitivity by enabling the use of a larger sample and allowing multiplication of the target organism in the medium. Generally an overnight incubation is all that is required for the desired sensitivity increase (Wernars, *et al.* 1991; Mahon, *et al.* 1994; Fach, *et al.* 1995). For example, Cherrington and Huis In't Veld (1993) developed an ELISA that was specific for *Salmonella typhimurium*, however the sensitivity of the technique was only 10^6 CFU ml^{-1}. By placing the sample in Muller-Kauffmann tetrathionate broth for 24 hours before performing the ELISA, positive ELISA reactions were achieved for samples that contained as few as 3 cells of *S. typhimurium* prior to enrichment. Other bacteria which have been detected by the combination of enrichment culture and ELISA techniques include: *Escherichia coli* (Todd, *et al.* 1988; Law, *et al.* 1992); *Listeria monocytogenes* (Heisick, *et al.* 1989; Palumbo 1991; Flanders, *et al.* 1995) and *Renibacterium salmoninarum* (Gudmundsdottir, *et al.* 1993).

In recent years enrichment culture has been widely used to enhance the sensitivity of PCR protocols (Fitter, *et al.* 1992; Giesendorf, *et al.* 1992; Gustafson, *et al.* 1992; Niederhaauser, *et al.* 1992; Hernandez, *et al.* 1995; Chiu and Ou 1996; Witham, *et al.* 1996; Bhaduri and Cottrell 1998; Fach, *et al.* 1999; Lindqvist 1999; Penyalver, *et al.* 2000; Vuddhakul, *et al.* 2000; Gonzalez-Rodriguez, *et al.* 2002; Cui 2003). As an example, Theron, *et al.* (2000) used selective-enrichment PCR for the sensitive detection of *Vibrio cholerae* from water samples. Without enrichment the sensitivity of the PCR procedure was 1.3×10^5 CFU ml^{-1} but after 6 hours

enrichment the detection limit was greatly enhanced with 390 CFU ml^{-1} (3.9 CFU per reaction) detected by PCR.

Further enhancements to the specificity of selective-enrichment PCR can be made by adding a hybridization step to the protocol. A specific probe can be used to internally verify the sequence of the PCR amplicon. Adding this step to the protocol also simplifies visualisation of the amplicon when performing high-throughput sampling. After PCR, small numbers of samples can be quickly and easily visualised by gel electrophoresis, but when large numbers of samples are being processed electrophoresis becomes cumbersome and time-consuming. Hybridization with an internal probe can be performed in a 96-well dot-blot apparatus allowing for high-throughput sampling and visualisation by colour formation on a membrane. However, even more convenient and often significantly more sensitive is micro-well hybridization, sometimes called PCR-ELISA but as the test is not immunological it is perhaps more accurately called a PCR- enzyme hybridization assay (EHA). In an EHA, PCR amplicons that have been generated with specially labelled primers are bound to the surface of a microtitre tray, a labelled probe is then hybridized to the bound amplicon which is then detected using one of the methods discussed in the 'Probe hybridization' section of this chapter. The most common PCR-EHA method uses biotin labelled primers during PCR and streptavidin coated microtitre trays for EHA, see Figure 1.2. Selective enrichment culture (SEC) PCR-EHA technology has been successfully applied to the detection of low-level infections in the food industry (Cocolin, *et al.* 2000; Bolton, *et al.* 2002), Antolin, *et al.* (2001) detected as little as 10 CFU ml^{-1} *Arcobacter* spp. and Grennan, *et al.* (2001) detected 40 CFU ml^{-1} *Campylobacter* spp. cells from poultry samples. Before the research reported in this thesis commenced, SEC-PCR-EHA technology had not been used for the detection of bacterial fish pathogens.

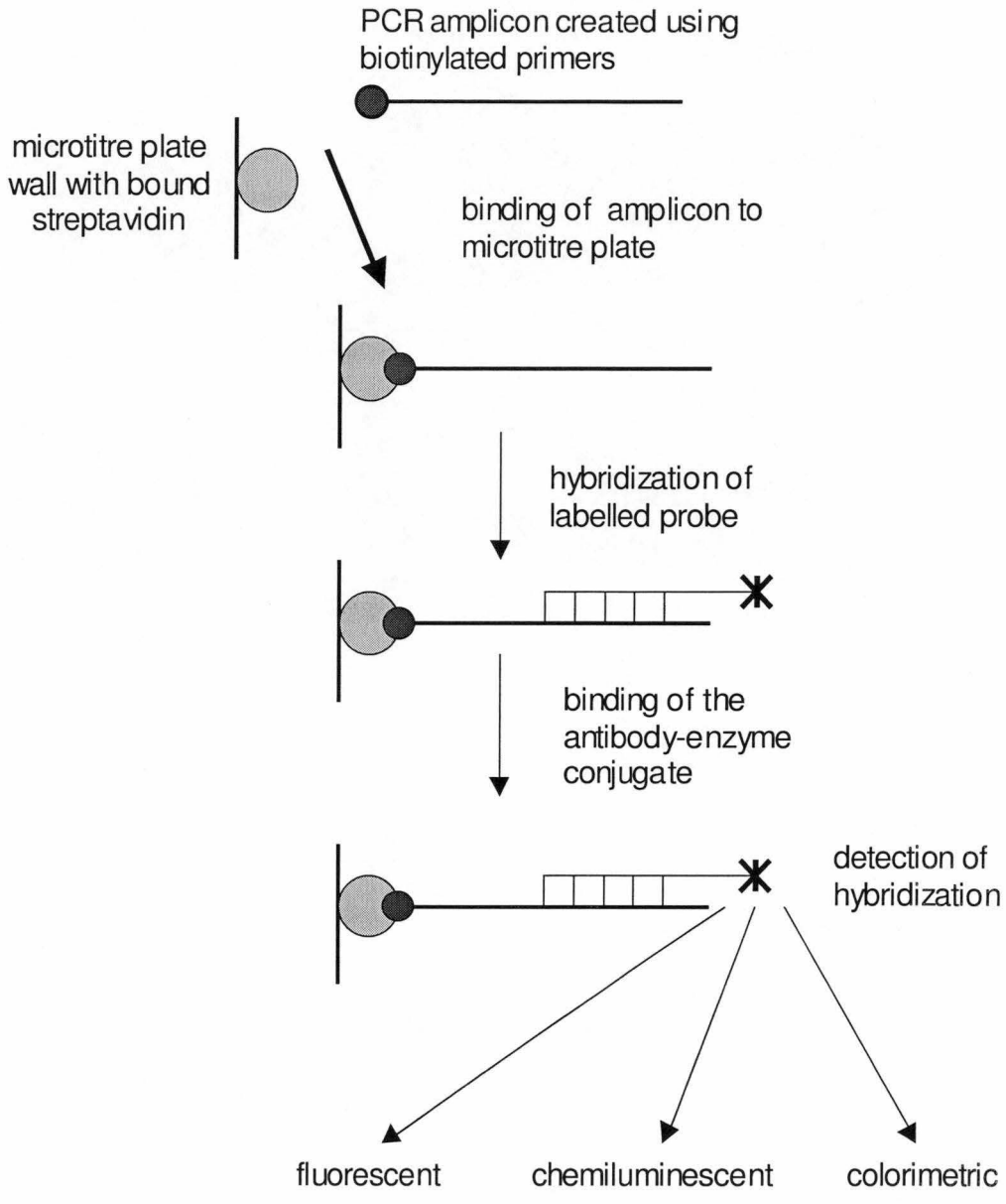


Figure 1.2: Detection of PCR amplicon with internal labelled primer using streptavidin coated microtitre trays for solid support.

This study details the development of SEC-(RT-)PCR-EHA for the detection of the four major bacterial pathogens *Aeromonas salmonicida; Lactococcus garvieae*; *Tenacibaculum maritimum* and *Yersinia ruckeri* in covertly infected farmed salmonid fish. The detection of covertly infected fish is essential for disease control, as through these fish the bacterium is actively spread to unaffected fish, increasing the percentage of carriers in the population or infecting previously unaffected stock. As the system is intended for use in surveillance and monitoring programs, large numbers of fish may need to be sampled at the one time. To facilitate this, a streamlined 96-well high-throughput sample processing method is used from the extraction of nucleic acids to the visualisation of the final results. In this form of miniaturisation only small amounts of reagents are used which helps to make the system low-cost and sample processing is performed using a multichannel pipette making the system rapid. During the SEC step of the system a 10 ml volume is used for each sample, miniaturisation is not used at this stage of the process as the larger volume enables a larger sample of tissue, which significantly increases the likelihood of detecting very low-level infections.

Laboratory development and validation of the system is conducted using the framework suggested by Hiney and Smith (1998b) and validation of the system with field samples is conducted from an epidemiological perspective using suggestions made by Dr Chris Baldock (AusVet Animal Health Services, Brisbane, pers. comm.). This approach is taken to ensure that validation of the system is an on-going process therefore resulting in a technology that is reliable and robust.

The first step in the development process is the optimisation of existing PCR protocols to produce the best possible sensitivity and specificity and the development of new sensitive RT-PCR protocols. As the system is intended for use in quarantine and surveillance programs, the prevention of false-positive reactions is critical. To help prevent these reactions an amplicon inactivation protocol is implemented. To enable high-throughput nucleic acid extraction, a 96-well vacuum system that uses the lysing and nuclease-inactivating properties of guanidinium isothiocyanate (GuSCN) and the DNA-binding properties of silica is developed. Following this, micro-well EHA technology is added to the system and

internal hybridization is used to verify the amplicon sequence, further enhancing the specificity of the protocol. Also, the micro-well EHA system greatly simplifies visualisation of the target PCR amplicons, allowing for the use of an ELISA plate reader to obtain results. The last step of the development process is the testing of system with field samples. To determine the performance of the system with field samples, test specificity and sensitivity were assessed from an epidemiological perspective. That is, specificity was determined using fish from farms with and without a history of disease, and sensitivity was determined by comparing the detection limit achieved in the laboratory using purified nucleic acids with that achieved using retrospective seeding of field samples. During the field trials large numbers of fish are tested and prevalence data is collected.

CHAPTER 2: Optimisation and Development of Background Technology.

INTRODUCTION:

Before embarking on the development of the SEC-PCR-EHA technology it was necessary to obtain a large supply of pure DNA from the target organisms to be used for positive controls and reference DNA. The DNA had to be free of PCR inhibitors so that the sensitivity achieved using this DNA represented the best possible sensitivity. The DNA had to be extracted in a high enough concentration to be stable for at least three years. For these reasons a large volume of media was used to grow each bacterium and a modified Marmur method (Marmur 1961) was used to obtain a large amount of high concentration, pure bacterial genomic DNA from the four test bacteria.

As it was intended that the SEC-PCR-EHA technology would use PCR primer sets developed by Carson (1998), optimisation of these primers to produce the best possible sensitivity and specificity was required. Optimisation was achieved by altering the annealing temperatures, the number of PCR cycles and the DNA polymerase from the original protocols given by Carson (1998).

After optimisation of the PCR protocols, RT-PCR protocols were developed. RT-PCR procedures were necessary to detect RNA from the target pathogens. RNA is more labile than DNA therefore RT-PCR gives a more accurate indication of the presence of live bacteria (Rosenthal and Landolo 1970), and therefore live covert infection. Also RT-PCR protocols often have a higher sensitivity than DNA PCR.

It was intended than internal hybridization probes would be used as an internal specificity check of any resulting PCR amplicon. Before embarking on the EHA it was decided that 'proof of concept' would be achieved by using Southern and dot blot techniques.

During the course of the study a new type of atypical *Aeromonas salmonicida*, named *A. salmonicida* biovar acheron, was isolated from Atlantic salmon in Tasmania. This biovar did not grow efficiently in the selective-

enrichment media that had been previously developed for *A. salmonicida*. Therefore an efficient selective medium to efficiently detect small numbers of *A. salmonicida* biovar acheron from farmed Atlantic salmon was developed.

TEST ORGANISMS AND PROBES:

Test bacteria

Aeromonas salmonicida DPIWE accession number 93/0956-2; *Lactococcus garvieae* ATCC 49156^T; *Tenacibaculum maritimum* NCIMB 2154^T and *Yersinia ruckeri* serotype O1b, DPIWE accession number 90/3988 were obtained from the culture collection held by the Fish Health Unit, Department of Primary Industries, Water and Environment, Tasmania (DPIWE). These organisms were the reference and positive control organisms for the entire study.

16S rRNA primers and internal probes

16S rRNA primer sets developed by Carson (1998) were used for the PCR assays, these are detailed in Table 2.1.

The internal biotin labelled probes were developed by Carson (1998), except the *A. salmonicida* probe which was described by Høie, *et al.* (1997). The sequences for these probes are given in Table 2.1. Melting temperatures of the labelled probes as calculated by the nearest neighbour method were 58°C for *L. garvieae*, 61°C for *Y. ruckeri* and 56°C for *T. maritimum* and *A. salmonicida*.

Table 2.1: PCR primer and internal probe sequences for the bacterial pathogens *A. salmonicida*, *L. garvieae*, *T. maritimum* and *Y. ruckeri*.

Species	Probe/ Primer	Probe acronym	Probe sequence 5'→ 3'
A. salmonicida	Forward PCR primer	AsF	TTT CGC GAT TGG ATG AA
	Reverse PCR primer	AsR	TTG ACA CGT ATT AGG CGC CA
	Internal hybidization probe	IntAs	GGA GGC AGC AGT GGG GAA TA
L. garvieae	Forward PCR primer	LgF	CGA GCG ATG ATT AAA GAT AGC TTG CTA
	Reverse PCR primer	LgR	ATA AGA ATC ATG CGA TTC TCA
	Internal hybidization probe	IntLg	TTT TTA TGA AGA GCG GCG AAC GGG T
T. maritimum	Forward PCR primer	TmF	GGA ATG GCA TCG TTT TAA AG
	Reverse PCR primer	TmR	AAT ACC TAC TCG TAG GTA CG
	Internal hybidization probe	IntTm	AAA GTT AAA GAT TTA TCG GTA GAA GAT GAC TAT
Y. ruckeri	Forward PCR primer	YrF	AAC CCA GAT GGG ATT AGC TAG TAA
	Reverse PCR primer	YrR	GTT CAG TGC TAT TAA CAC TTA ACC C
	Internal hybidization probe	IntYr	GCA CTT TCA GCG AGG AGG AAG GGT TAA

2.1. DEVELOPMENT OF PCR AND RT-PCR PROTOCOLS

MATERIALS AND METHODS:

Large scale extraction of pure genomic DNA

Pure DNA was extracted from the four bacteria using a method based on Marmur (1961) with some modifications due to the mucoid nature of *T. maritimum* and Gram-positive cell wall of *L. garvieae*. This method is described below.

Preparation of cells.

A starter culture of 10 ml Brain Heart Infusion broth (BHI) (Oxoid, UK) for *L. garvieae*, modified Brain Heart Infusion broth for fastidious *A. salmonicida* (Cooperative Research Centre for Aquaculture Ltd, Sydney, proprietary formulations), tryptone soya broth (TSB) (Oxoid) for *Y. ruckeri* or marine Sheihs broth (Appendix A) for *T. maritimum* was prepared and incubated at 25°C until turbid. Starter cultures were used to inoculate one litre of broth, which was incubated at 25°C until very turbid. For *L. garvieae* the BHI was supplemented with 2 g L^{-1} DL-threonine (Sigma-Aldrich, Missouri, USA) (Klaenhammer *et al.* 1978) and after 12-18 hours a sub-lethal concentration of 0.12 g L^{-1} penicillin G (Sigma-Aldrich) (Komatsu 1979) was added to weaken the cell wall allowing easy access to the DNA later in the procedure. For *T. maritimum* the broth was stirred and aerated to encourage dense and even bacterial growth. Purity of each bacterium was checked by subculture.

The cells were pelleted by centrifugation at 3900*g* for 20 minutes at 10°C. For *T. maritimum*, the pellet was washed in 10 ml 0.1M saline-EDTA (Appendix A) and 20 ml ice cold ethanol, vortexed, and the volume made up to 50 ml with saline-EDTA and centrifuged again (L. Schmidtke pers. comm.). For the other bacteria the pellet was resuspended in 25 ml total saline-EDTA and centrifuged and the weight of the cells checked to determine if between 0.5 and 1 g.

Lysis of cells and extraction of DNA.

The pellet was resuspended in 8 ml 0.1 M saline-EDTA to give an even suspension free from clumps. Then 1 ml of 20 mg ml^{-1} lysozyme (Appendix A) was added, the suspension vortexed and incubated for 1 hour at 37°C, or overnight for *L. garvieae*. After lysozyme treatment 1 ml of 10% (w/v) SDS and 20 µl of 50 mg ml^{-1} proteinase K (Appendix A) (Keller and Manak 1989) was added and the cells incubated at 50°C for 30 minutes in a water-bath. A further 0.5 ml of 10% (w/v) SDS was added to the cells followed by 2, 5 minute 50°C incubation steps with cooling to 4°C between each incubation. At this stage a drop of cells was examined by phase microscopy at 40X to determine the extent of cell lysis. If the bacterial cells were still largely intact short incubation steps at 55°C followed by rapid cooling was repeated until cell lysis occurred.

After cell lysis, 2.75 ml of 5 M sodium perchlorate was added to the cells and shaken for 10 minutes at room temperature. The cell debris was separated from the nucleic acids by adding one volume of biotechnology grade chloroform/isoamly alcohol 24:1 (Amresco, Ohio, USA), shaking for 30 minutes at room temperature and centrifuging at 3000g for 15 minutes. The top layer was carefully collected using a wide bore pipette and overlayed with two volumes of cold absolute ethanol and the nucleic acids collected by spooling with a glass rod. Any residual ethanol was allowed to evaporate and the nucleic acids were dissolved in 5 ml 10 mM Tris, pH 8 (Amresco).

After no visible signs of nucleic acids could be seen on the glass rod it was removed and the solution warmed to 37°C for 15 minutes to completely dissolve the nucleic acids. RNA was hydrolysed by incubating the solution for 45 minutes at 37°C with 250 µl of 0.2% RNAase prepared in 0.1M Tris pH 8.0 (Keller and Manak 1989). A volume of 5 ml phenol equilibrated to pH 8 (Amresco) was then added, the solution shaken for 10 minutes at room temperature, followed by centrifugation at 1500g for 10 minutes. The upper aqueous layer was then collected using a wide-bore pipette; over-layed with 2 volumes of cold absolute ethanol and the DNA spooled using a glass rod. Any residual ethanol was allowed to evaporate and the DNA dissolved in 10 mM Tris, pH 8. One volume of chloroform/isoamyl alcohol 24:1 was added to the DNA, shaken for 15 minutes at room temperature, followed by centrifugation at 3000g for 15 minutes. The upper aqueous layer was collected and the chloroform/isoamly alcohol procedure repeated. The upper aqueous layer was collected one last time and over-layed with two volumes of cold absolute ethanol and pure DNA spooled using a glass rod. Any residual ethanol was allowed to evaporate and the DNA was dissolved in 5 ml 10 mM Tris, pH 8 overnight at 4°C, the glass rod was removed and the DNA stored at -20°C.

Quantity and quality of DNA.

In order to give an indication of the extent of DNA shearing and the completeness of RNA hydrolysis, the DNA samples were assayed by electrophoresis on a 1% (w/v) agarose gel containing 0.5 µg ml^{-1} ethidium bromide (Sigma-Aldrich) prepared in TAE buffer (Appendix A). The gels were

run using a horizontal gel electrophoresis apparatus (Horizon 58, Invitrogen) at 70 V for 40 minutes. In each case a BresaGen (Thebatron, SA, Australia) SPP-1 (digested with *Eco* RI restriction enzyme) DNA ladder was run alongside the DNA.

The final concentration of pure DNA was determined using a Bio-Rad Versafluor Fluorimeter and a Hoescht H33258 DNA specific dye. Zero calibration values were set using Tris-Saline-EDTA and Hoescht dye and 100 ng μl^{-1} point determined using Tris-Saline-EDTA, Hoescht dye and a calf thymus DNA standard. The DNA samples were measured by adding 2 µl of sample to 2000 µl of Tris-Saline-EDTA plus Hoescht dye.

The quality of the DNA was measured by the A_{260}/A_{280} ratio using a Genequant DNA quantifier (Pharmacia Biotech, Cambridge, UK).

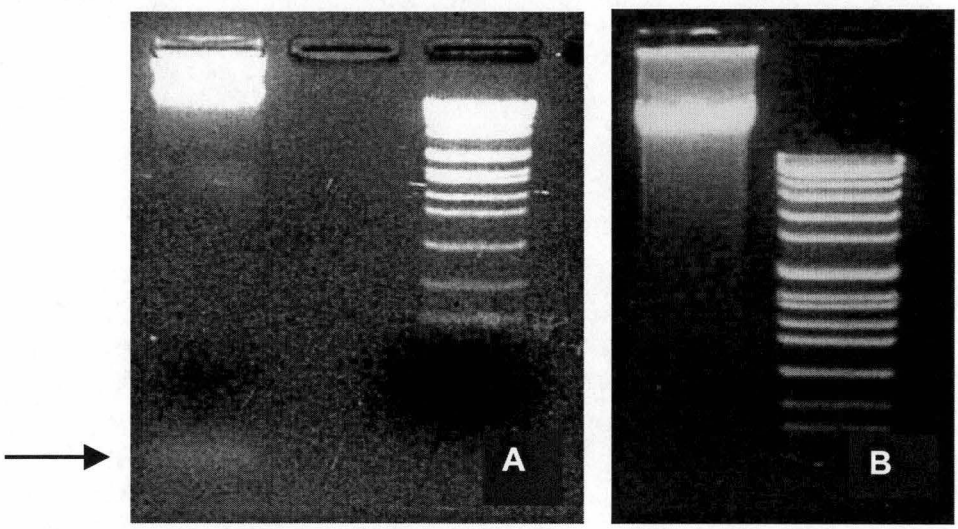


Figure 2.1: 1% agarose gel electrophoresis of *Yersinia ruckeri* DNA before RNase treatment, arrow shows RNA [A] and after RNase treatment [B]. In each case the DNA ladder is BresaGen SPP-1 (digested with *Eco* RI restriction enzyme) DNA ladder.

Extraction of pure ribosomal RNA

Pure RNA was extracted using an RNAAqueous^{TM}-4PCR (Ambion, Austin, Texas, USA) extraction kit. Briefly:

Purity plates of each bacterium were prepared using Blood Agar Base No. 2 (Oxoid) supplemented with 7% (v/v) defibrinated sheep's blood (SBA). From these plates dense suspensions of bacteria were prepared in 200 µl of 18 Mohm water. The samples were then pelleted by centrifuging at 15000g for 5 minutes. A 200 µl volume of lysis/binding solution (Ambion) was added to the pellets and the samples vigorously vortexed until the lysate was homogenous.

A 200 µl volume of 64% ethanol was added and the samples mixed by inverting the tube 6 times. The mixtures were then transferred to the proprietary filter cartridges and the samples centrifuged at 15000*g* for 1 – 3 minutes (until the lysate had passed through the filter). The flow-through was discarded and 700 µl of Wash solution #1 (Ambion) was added to each filter cartridge. The samples were centrifuged again and the flow-through discarded. A volume of 500 µl Wash solution #2/3 was added to the filter cartridge, the samples centrifuged and then the wash step repeated. The filter cartridge was then placed inside a collection tube and 40 µl of hot (95 - 100 °C) Elution solution (Ambion) was added to the filter cartridge. The samples were again centrifuged at 15000*g*. A second aliquot of Elution solution, this time only 10 µl, was added to the samples and centrifugation repeated. The eluted liquid was collected.

The quality of the RNA was measured by the A_{260}/A_{280} ratio using a Genequant DNA quantifier (Pharmacia Biotech).

Optimisation of existing PCR protocols

PCR optimisation was achieved in three ways, by the choice of *Taq* polymerase, by varying the concentration of magnesium chloride and by altering the PCR cycling conditions such as annealing temperatures and PCR extension times to suit each bacterium. To test the effectiveness of these variations decimal dilutions of the purified DNA were prepared in sterile 18 Mohm water and PCR was performed until maximum sensitivity was achieved without compromise to specificity. Specificity was assessed at two levels: firstly, using phenotypically similar bacteria, near related species and bacteria likely to be isolated alongside the target bacterium; secondly using bacteria identified as having some genotypic similarity in respect to the specific

16S rRNA primer sequence as identified by Carson (1998). The bacteria tested for each of the four bacteria are listed in Table 2.2.

All results were visualized by gel electrophoresis (70 V for 40 minutes) on a 2% (w/v) agarose gel prepared in TAE buffer and containing 0.5 μg ml^{-1} ethidium bromide (Sigma-Aldrich).

Taq efficiency.

The different enzymes tested were: HotStar *Taq* polymerase (Qiagen, Germany); Red Hot DNA polymerase (Advanced Biotechnologies, UK) and Platinum *Taq* DNA polymerase (Invitrogen, CA, USA). The enzymes were tested in the PCR at the concentration recommended by the supplier and at half that concentration.

Magnesium chloride concentration.

The concentrations of magnesium chloride tried in the PCR reaction were 1 mM, 1.5 mM, 2 mM, 2.5 mM and 3 mM.

PCR cycling conditions.

For each bacterium the annealing temperatures trialed were 55°C, 58°C, 60°C, 62°C and 65°C. The extension times trialed were 2 minutes, 1 minute, 45 seconds and 30 seconds.

Development of RT-PCR protocols

Before attempting to develop the RT-PCR all reagents were made RNase-free. This was achieved by treating all solutions with 0.2% (v/v) diethyl pyrocarbonate (DEPC), allowing the solutions to stand overnight at room temperature followed by autoclaving at 121°C for 30 minutes.

Because of the high level of sensitivity achieved using Platinum *Taq* DNA polymerase (Invitrogen) for PCR, Superscript™ One-Step RT-PCR with Platinum *Taq* (Invitrogen) was used for the RT-PCR assays.

RT-PCR optimisation was achieved in three ways, by varying the concentration of magnesium sulphate, varying the method of pre-RT-PCR DNase treatment and altering the PCR cycling conditions such as annealing

temperatures and PCR extension times to suit each bacterium. To test the effectiveness of these variations decimal dilutions of the RNA extracted using the Ambion extraction kit were prepared in sterile DEPC treated 18 Mohm water and RT-PCR was performed until maximum sensitivity was achieved without compromise to specificity. Two negative controls were used in every RT-PCR assay, a negative control that contained RT-PCR with Platinum *Taq* enzyme but no RNA template and a negative control that contained RNA template but no RT-PCR with Platinum *Taq* enzyme. Specificity was assessed using the bacteria listed in Table 2.2.

All results were visualized by gel electrophoresis (70 V for 40 minutes) on a 2% (w/v) agarose gel containing 0.5 µg ml^{-1} ethidium bromide (Sigma-Aldrich) prepared in TAE buffer.

Magnesium sulphate concentration.

The concentrations of magnesium sulphate tried in the PCR reaction were 1.2 mM, 1.5 mM, 1.8 mM, 2.0 mM, 2.5 mM and 3 mM.

PCR cycling conditions.

For each bacterium the annealing temperatures trialed were 58°C, 60°C, 62°C and 65°C. The extension times tested were 1 minute, 45 seconds and 30 seconds.

DNase treatment.

After RNA extraction the samples were treated with DNase 1 (Promega, USA) to remove all traces of DNA that may have been left in the sample. Different DNase treatment conditions were tested as the method described in the RNAAqueous^{TM}-4PCR manual (Ambion) did not appear to work with a positive (DNA) band remaining in the negative control. DNase was tried at concentrations of 0.1 U, 0.2 U and 0.4 U per µl of sample. Manganese chloride was tested as an alternative cofactor for the reaction, instead of magnesium chloride. Using a 'stop reagent' (Promega) and heating the sample after DNase treatment were tested as alternative ways of stopping the DNase reaction.

Table 2.2: Bacteria used for testing specificity of PCR and RT-PCR protocols.

Species	Strain no.	Homology[a]
***Aeromonas salmonicida* specificity**		
A salmonicida[b]	84/09062-B13	100%
A sobria	ATCC 43979^T	100%
A eucrenophila	ATCC 23309^T	94%
A jandaei	ATCC 49568^T	94%
A schubertii	ATCC 43700^T	94%
A veronii bv *sobria*	ATCC 9071^T	94%
A veronii bv *veronii*	ATCC 35624^T	94%
A hydrophila	ATCC 7966^T	85%
A hydrophila	ATCC 7965	85%
A hydrophila[c]	UTS 67	85%
A bestiarum	ATCC 14715	85%
A caviae	ATCC 15468^T	76%
A trota	ATCC 49657^T	76%
A media	ATCC 33907^T	75%
Vagococcus salmoninarum	NCDO 2777^T	
Tenacibaculum maritimum	NCIMB 2154^T	
Hafnia alvei	95/6404	
Proteus rettgeri	96/5494	
Yersinia intermedia	92/4041	
Aeromonas sp [f]	NF 1	
Aeromonas sp [f]	NF 2	
Pseudomonas sp [f]	NF 3	
Enterobacter sp [f]	NF 4	
***Tenacibaculum maritimum* specificity**		
Cytophage marinoflava	ACAM 75	65%
T ovolyticum	NCIMB 13127^T	
FCLB mucoid[e]	89/2244-9	
FCLB mucoid[e]	89/2756-1	
FCLB mucoid[e]	96/5171	
FCLB mucoid[e]	CRC-2	
F columnare	NCIMB 2248^T	
F johnsoniae	ATCC 17061$^{Co\ T}$	
Vibrio splendidus biovar I	ATCC 25914^T	
V ordalii	ATCC 33509^T	
V anguillarum	85/3475-1	

Species	Strain no.	Homology[a]
***Lactococcus garvieae* specificity**		
Carnobacterium piscicola	ATCC 35586^T	76%
Flavobacterium columnare	NCIMB 2248^T	71%
Streptococcus sp [f]	CORT 1	
Streptococcus sp [f]	CORT 2	
L piscium	NCDO 2778^T	
S iniae[d]	95.41693/4A	
Enterococcus faecalis	ATCC 29212	
V salmoninarum	NCDO 2777^T	
A hydrophila	ATCC 7966^T	
***Yersinia ruckeri* specificity**		
Y pseudotuberculosis	96/5417-2	96%
Y enterocolitica	96/5440-1B	87 5%
Haemophilus influenzae	ATCC 33391^T	68%
Citrobacter freundii	90/2624-18	87 5%
Y intermedia	92/4041	
H alvei	95/6404	87 5%
Proteus rettgeri	96/5494	
V anguillarum	85/3475-1	
Carnobacterium piscicola	ATCC 35586^T	
V salmoninarum	NCDO 2777^T	
Pseudomonas sp [f]	NF 3	
Enterobacter sp [f]	NF 4	
Specificity: All systems		
A salmonicida	93/0956-2	
Escherichia coli	ATCC 25922	87.5% (*Y. ruckeri*)
L garvieae	ATCC 49156^T	
Y ruckeri serotype O1b	90/ 3988	

[a] Percentage homology is in respect of the primer of closest match.

[b] Atypical *A. salmonicida* isolated from goldfish in Victoria, strain supplied by N. Gudkovs, Australian Fish Disease Laboratory, Australian Animal Health Laboratory, CSIRO, Australia.

[c] Strain supplied by J. Oakey, University of Technology, Sydney, Australia.

[d] Strain supplied by A. Thomas, Queensland Department of Primary Industries, Australia.

[e] FCLB: Unidentified *Flexibacter-Cytophaga*-like normal flora isolated from Atlantic salmon.

[f] Fish normal flora isolated from rainbow trout or Atlantic salmon.

RESULTS:

Extraction of nucleic acids

The concentration of DNA and the A_{260}/A_{280} ratios for each bacterium are given in Table 2.3. As highly concentrated solutions are more stable when frozen, the concentrations achieved here (> 110 ng μl^{-1}) should mean that the DNA is stable when frozen at –20 °C for many years. The quality of the extracted nucleic acids was high with A_{260}/A_{280} ratios of greater than 1.6 except for the *L. garvieae* which gave a ratio of only 1.3. By electrophoresis, the DNA extracted from each of the bacteria was strong producing a bright clear band and no RNA smear after RNase treatment. Figure 2.1 shows the results achieved for *Y. ruckeri* before and after RNase treatment. Similar results were achieved for the other three bacteria.

High quality RNA was produced using the RNAAqueous^{TM}-4PCR (Ambion) extraction kit, the A_{260}/A_{280} ratios were always between 1.95 and 2.1 and the concentration of RNA usually about 30 ng μl^{-1}. Large quantities of concentrated RNA were not produced, instead new RNA extractions were produced every three months or whenever the stock ran out.

PCR optimisation

Polymerase trials were conducted with *L. garvieae* and *Y. ruckeri* DNA, using the PCR parameters published by Carson (1988). Platinum *Taq* DNA polymerase (Invitrogen) produced the most sensitive results with a template concentration of 54 fg for *L. garvieae* and 240 fg of *Y. ruckeri* DNA detectable by electrophoresis. The HotStar *Taq* polymerase (Qiagen) was almost as sensitive as the Platinum *Taq*, producing a weak band from 160 fg of *L. garvieae* and 480 fg of *Y. ruckeri* DNA, however this enzyme was more expensive to purchase. The Red Hot DNA polymerase (Advanced Biotechnologies) used by Carson (1988) was significantly less sensitive than the other enzymes, see Table 2.5 and Figure 2.2.

Raising the concentration of magnesium chloride did not increase the sensitivity of the PCR assays significantly. However this increase in concentration did decrease the fidelity of the PCR with spurious bands visible

by electrophoresis. This was particularly noticeable when testing the *A. salmonicida* PCR for specificity, see Figure 2.3.

Initial PCR conditions as published by Carson (1998) are given in Table 2.5. Decreasing the annealing temperatures greatly increased the PCR sensitivity. However if the annealing temperatures were decreased by too much, some closely related species gave positive PCR reactions. Therefore, the annealing temperatures were decreased as much as specificity restrictions would allow. Figure 2.4 shows the increase in sensitivity achieved for *Y. ruckeri* when the annealing temperature was decreased from 65°C to 60°C. The annealing temperature for the *L. garvieae* and *T. maritimum* PCR was decreased by 3°C, the temperature for *Y. ruckeri* was decreased by 5°C. The annealing temperature for *A. salmonicida* could not be decreased, however greatly improved sensitivity was achieved by increasing the number of PCR cycles from 30 to 35. After optimisation the specificity achieved for the 16S primer sets for each bacterium by Carson (1998) was not compromised.

The optimised PCR conditions and the resulting sensitivity values are given in Table 2.5.

RT-PCR development

Similar sensitivity values were achieved for the RT-PCR protocols as those achieved for the PCR protocols, see Table 2.5. As for the PCR, sensitivity was not increased by the addition of more magnesium to the reaction mix. Initially it was impossible to obtain an accurate sensitivity value for the RT-PCR as the titration endpoints were obscured by the presence of weak bands in the no RT enzyme negative control (no reverse transcriptase enzyme but with template and *Taq* polymerase). This problem was rectified by using manganese chloride (Sigma-Aldrich) instead of magnesium chloride when treating the nucleic acids with DNase pre-RT-PCR (Figure 2.5). The optimum DNase concentration was 0.1 U per µl of total volume, for example for 3 µl of RNA template the mixture would contain 0.4 µl of DNase 1 (Promega), 0.4 µl 10 nM $MnCl_2$ (Appendix A) and 0.16 µl of 10mM $CaCl_2$ / 900mM Tris-HCl, pH 8.0 (Appendix A). The optimum DNase and buffers for three different sample volumes are given in

Table 2.4. Optimum DNase conditions were activation at 37°C for 30 minutes, denaturation at 75°C for 5 minutes followed by cooling to 4°C before use.

Table 2.3: Concentration and quality of DNA produced from large-scale DNA extraction.

Bacterium	**DNA concentration**	**A_{260}/A_{280} ratio**
Aeromonas salmonicida	111 ng μl^{-1}	1.7
Yersina ruckeri	146 ng μl^{-1}	1.7
Lactococcus garvieae	670 ng μl^{-1}	1.3
Tenacibaculum maritimum	685 ng μl^{-1}	1.6

Table 2.4: Optimum DNase and activation cofactor ($MgCl_2$) volumes for three different sample sizes.

Ingredient	**3µl template**	**5µl template**	**10µl template**
Promega DNase (1 U μl^{-1})	0.4 µl	0.5 µl	1.0 µl
10mM $MnCl_2$ (Appendix A)	0.4 µl	0.63 µl	1.3 µl
10mM $CaCl_2$ / 900mM Tris-HCl, pH 8.0 (Appendix A)	0.16 µl	0.25 µl	0.5 µl
Total	0.96 µl	1.38 µl	2.8 µl

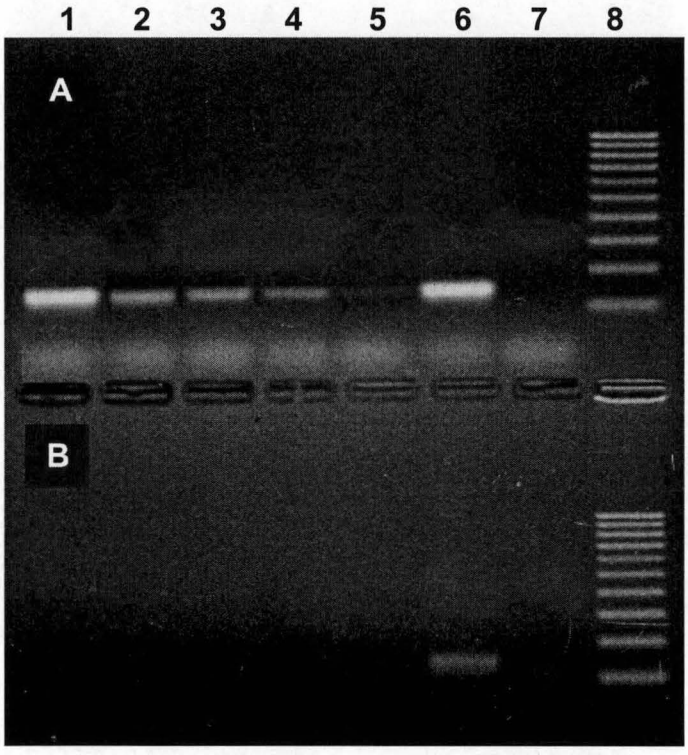


Figure 2.2: Sensitivity of *Lactococcus garvieae* PCR with row [A] Platimun *Taq* DNA polymerase and row [B] Red Hot DNA polymerase. Lane 1, 5 pg μl^{-1}; lane 2, 1 pg μl^{-1}; lane 3, 200 fg μl^{-1}; lane 4, 40 fg μl^{-1}; lane 5, 8 fg μl^{-1}; lane 6 10 ng μl^{-1} positive control; lane 7, negative control; lane 8, Advanced Biotechnologies 100 bp ladder.

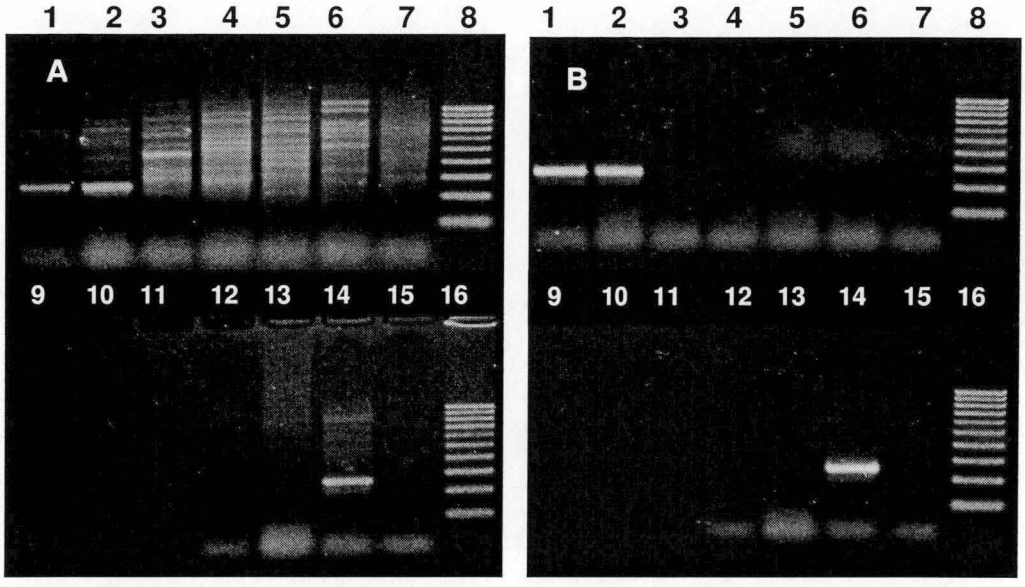


Figure 2.3: Specificity of *Aeromonas salmonicida* PCR with [A] 2 mM magnesium chloride and [B] 1.375 mM magnesium chloride. Lane 1, *A. bestiarum* ATCC 14715; lane 2, *A. hydrophila* ATCC 7965; lane 3, *A. caviae* ATCC 15468^{T}; lane 4, *A. eucrenophila* ATCC 23309^{T}; lane 5, *A. sobria* ATCC 43979^{T}; lane 6, *A. media* ATCC 33907^{T}; lane 7, *A. veronii* bv *sobria* ATCC 9071^{T}; lane 8, Advanced Biotechnologies 100 bp ladder; lane 9, *A. veronii* bv *veronii* ATCC 35624^{T}; lane 10, *A. schubertii* ATCC 43700^{T}; lane 11, *A. trota* ATCC 49657^{T}; lane 12, *A. jandaei* ATCC 49568^{T}; lane 13, *Hafnia alvei* Accession# 95/6404; lane 14, *A. salmonicida* 93/0956-2; lane 15, negative control; lane 16, Advanced Biotechnologies 100 bp ladder.

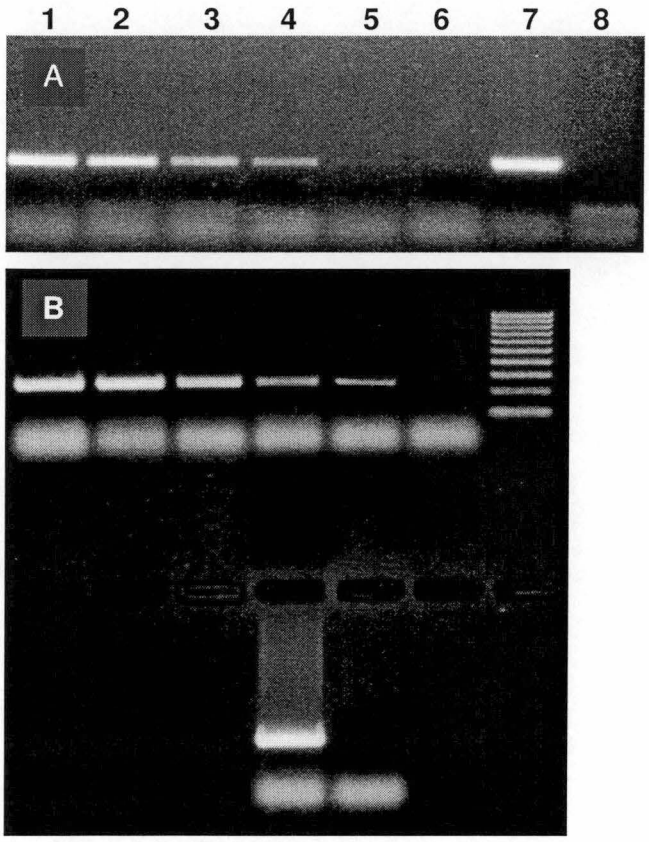


Figure 2.4: Sensitivity of *Yersinia ruckeri* PCR [A] with an annealing temperature of 65°C: lane 1, 30 pg μl^{-1}; lane 2, 6 pg μl^{-1}; lane 3, 1.2 pg μl^{-1}; lane 4, 240 fg μl^{-1}; lane 5, 48 fg μl^{-1}; lane 6, 9.6 fg μl^{-1}; lane 7, 10 ng μl^{-1} positive control; lane 8, negative control. [B] with an annealing temperature of 60°C, row 1: lane 1, 10 pg μl^{-1}; lane 2, 1 pg μl^{-1}; lane 3, 200 fg μl^{-1}; lane 4, 40 fg μl^{-1}; lane 5, 8 fg μl^{-1}; lane 6, ~ 1 fg μl^{-1}; lane 7, Advanced Biotechnologies 100 bp ladder. Row 2: lane 4, 10 ng μl^{-1} positive control; lane 5, negative control.

Table 2.5: PCR conditions before and after optimisation.

Bacterium	Cycle conditions by Carson 1998	Optimum cycle conditions
Aeromonas salmonicida	Red Hot polymerase 1.375 mM $MgCl_2$ 30 cycles 60°C annealing temp 2 min extension time Sensitivity: 10 pg using nested PCR	Platinum *Taq* DNA polymerase 1.375 mM $MgCl_2$ 35 cycles 60°C annealing temp 30 sec extension time Sensitivity: 4 fg For RT-PCR: Superscript™ One-Step RT-PCR with P*Taq* Sensitivity: 4 fg
Lactococcus garvieae	Red Hot polymerase 2 mM $MgCl_2$ 35 cycles 65°C annealing temp 2 min extension time Sensitivity: 1 ng	Platinum *Taq* DNA polymerase 2 mM $MgCl_2$ 35 cycles 62°C annealing temp 30 sec extension time Sensitivity PCR: 4 fg For RT-PCR: Superscript™ One-Step RT-PCR with P*Taq* Sensitivity: 280 ag
Tenacibaculum maritimum	Red Hot polymerase 2 mM $MgCl_2$ 35 cycles 65°C annealing temp 2 min extension time Sensitivity: 1 pg	Platinum *Taq* DNA polymerase 2 mM $MgCl_2$ 35 cycles 62°C annealing temp 30 sec extension time Sensitivity PCR: 4 fg For RT-PCR: Superscript™ One-Step RT-PCR with P*Taq* Sensitivity: 40 fg
Yersinia ruckeri	Red Hot polymerase 2 mM $MgCl_2$ 35 cycles 65°C annealing temp 2 min extension time Sensitivity: 10 pg	Platinum *Taq* DNA polymerase 2 mM $MgCl_2$ 35 cycles 60°C annealing temp 30 sec extension time Sensitivity PCR: 4 fg For RT-PCR: Superscript™ One-Step RT-PCR with P*Taq* Sensitivity: 4 fg

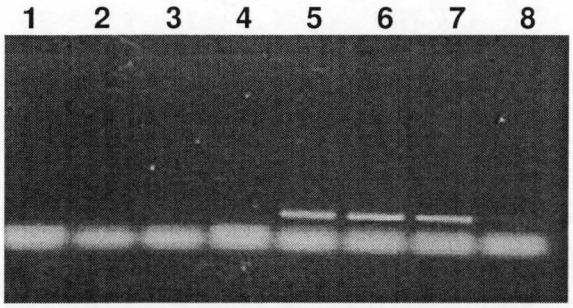


Figure 2.5: Replicate *Y. ruckeri* RT-PCR negative controls (no Superscript™ One-Step RT-PCR enzyme, with Platinum *Taq* DNA polymerase and DNase treated template). Lanes 1 to 4 DNase treatment before PCR using using manganese chloride, Lanes 5 to 8 DNase treatment before PCR using magnesium chloride. Before DNase treatment each RT-PCR reaction contained approximately 10 ng of mixed RNA and DNA.

DISCUSSION:

Nucleic acid extraction

DNA extraction was uncomplicated for *A. salmoncida* and *Y. ruckeri* however the variations to the procedure for *T. maritimum* and *L. garvieae* were essential in achieving efficient DNA extraction. *T. maritimum* is strictly aerobic and because of its gliding nature it is not able to move freely through a liquid (Reichenbach and Dworkin 1981). When grown without stirring and aeration *T. maritimum* grew in an uneven suspension resulting in a light concentration of the bacterium and therefore a very low DNA concentration in the final extraction. Also, due to the 'sticky' nature of the cells *T. maritimum* was washed with saline-EDTA before cells lysis, increasing the efficiency of lysis. With the *L. garvieae*, DNA extraction was not efficiently achieved without the addition of DL-threonine and a sub-lethal concentration of penicillin G to weaken the Gram-positive cell wall allowing for efficient cell lysis using lysozyme later in the procedure.

Approximately 5 ml of concentrated DNA was produced for each bacterium. The DNA was stored at –20°C and over the three year life of the project was used to determine PCR sensitivity and as positive controls. During the three

years the DNA did not noticeably decline in concentration or quality. Pure DNA has an A_{260}/A_{280} ratio of 1.8-2.0 in 10 mM Tris.Cl, pH 8.5 (Sauer, *et al.* 1998), the ratio of the extracted DNA was slightly less than 1.8 and significantly less than this for *L. garvieae*, thus indicating that the DNA was not perfectly pure. A low ratio can indicate the presence of contaminants such as proteins or phenol (Sauer, *et al.* 1998), while the DNA was thoroughly washed with ethanol it is possible that traces of these contaminants could remain in the final product.

Pure RNA has an A_{260}/A_{280} ratio of 1.9-2.1 in 10 mM Tris.Cl, pH 7.5 (Sauer, *et al.* 1998) and as the RNA extracted using the Ambion RNAAqueous^{TM}-4PCR extraction kit always fell between these values high quality RNA was produced.

PCR and RT-PCR development and optimisation

With the detection of carriers of pathogens being the ultimate aim of the project, optimisation of the PCR and RT-PCR protocols to achieve maximum sensitivity was very important. The excellent PCR and RT-PCR sensitivity values achieved were only possible with the combination of improved *Taq* polymerase, and PCR cycling conditions. These sensitivity values were used as a goal for PCR and RT-PCR performed using nucleic acids extracted from selective-enrichment media. By quantifying the sensitivity, sub-optimal PCR due to the presence of inhibitors or inefficient nucleic acid extraction could be determined.

It was equally important to retain the integrity of the PCR specificity during optimisation; this limited the concentration of magnesium and the minimum annealing temperatures that could be used. This was not an unexpected result as increasing magnesium and decreasing annealing temperatures decreases the fidelity of PCR (Atlas and Bej 1994). For RT-PCR the use of manganese in the pre-RT-PCR DNase step was critical in avoiding false-positive RT-PCR reactions due to remnant DNA. Divalent metal ions are a cofactor necessary for the hydrolysis of DNA by DNase (Melgar and Goldthwait 1968). While it is usual to use magnesium to activate DNase, studies have shown that manganese increases the activity of DNase and produces DNA fragments with blunt ends that have less potential to recombine than do the overlapping fragments produced when using magnesium as a cofactor (Bauer, *et al.* 1997). A 'stop

reagent' was supplied with the Promega DNase, but was not used in the final RT-PCR protocol. While there was no evidence that this reagent did not work, when the DNase was activated at 37°C for 30 minutes and denatured at 75°C for 5 minutes (Huang, *et al.* 1996) the reagent was not required to stop the reaction. Also the 'stop reagent' contained EGTA which has the potential to chelate the magnesium ions in the RT-PCR reaction mix possibly resulting in sub-optimal sensitivity. Therefore, after optimisation PCR and RT-PCR specificity were not compromised beyond the limitations of the PCR primers and corresponding internal probes. That is, no cross-reaction was found with the *T. maritimum, L. garvieae* and *Y. ruckeri* PCR and RT-PCR protocols and, as expected, cross-reaction was found with some strains of *A. hydrophila* and *A. bestiarum* (Carson 1998) when performing the *A. salmonicida* PCR.

2.2. DEVELOPMENT OF HYBRIDIZATION PROTOCOLS

MATERIALS AND METHODS:

Test DNA

DNA from test organisms was extracted using Method 2.1.

PCR technique

The PCR reaction mix contained 200 µM each of dNTPs, 1.375 mM $MgCl_2$ for *A. salmonicida* and 2 mM $MgCl_2$ in all other cases, 1 X PCR buffer (Invitrogen), 2 µM each of the two primers, 0.5 units Platinum *Taq* DNA Polymerase (Invitrogen), 1 µl template DNA (40 ng μl^{-1} unless specified otherwise) and sufficient 18 Mohm water to bring the total reaction volume to 20 µl. The PCR cycling conditions used were those described in Method 2.2.

Southern blotting

PCR products were detected by electrophoresis on a 2% (w/v) agarose gel. After visualisation the gel was depurinated using 0.25 M HCl for 10 minutes, denatured in two stages using 0.5 M NaOH, 1.5M NaCl for a total of 30

minutes, and neutralised using 1.5 M NaCl, 0.5 M Tris HCl, pH 7.5 for 30 minutes with gentle agitation. The gel was then rinsed thoroughly in reverse osmosis water (RO <2 µS).

A capillary transfer as shown in the Figure 2.6 was set-up by first cutting a 'wick' out of 3 mm Whatman blotting paper and placing it on the 'support' with each end dipping into the 'troughs'. The 'troughs' were filled with transfer buffer and the 'wick' allowed to take up the liquid. Any air bubbles that developed under the wick were carefully smoothed out. The agarose gel was inverted and placed on the damp wick, again removing air bubbles. The ends of the gel were then surrounded with cling flim to prevent short-circuiting of fluid between the 3 mm wick and the paper towels. A scalpel blade was used to cut a piece of Immobilon -Ny+ Nylon membrane (Millipore, USA), about 1 – 2 mm larger than the gel in both dimensions. The membrane was hydrated in RO water (<2 µS) and then soaked in 10 x SSC (Appendix A) for 5 minutes. The wet membrane was gently positioned over the gel in the transfer box and air bubbles were gently removed. Two pieces of 3 mm paper cut to the same size as the gel and then soaked in 10 x SSC were positioned on top of the nylon membrane. A 5 - 10 cm stack of paper towels was then placed on top of the gel and 3 mm paper, and a 500 g weight was placed on top. Capillary transfer was allowed to proceed overnight at room temperature.

After Southern blotting, the membrane was carefully separated from the dehydrated gel. The membrane was then left to dry at room temperature for about 1 hour and then the DNA crosslinked to the membrane using 254 nm UV light at 5000 µJ cm^{-2} for 30 seconds using a UV crosslinker, Spectrolinker XL-1000 (Spectronics Corporation, New York, USA).

Dot blotting

Thirty microlitres of 2 x SSC was added to each tube that contained PCR product. The PCR products were placed in a thermal-cycler and denatured at 96°C for 10 minutes and then cooled rapidly to 4°C. Fifty microlitres of 20 x SSC was then added to each tube. An appropriate size of Immobilon -Ny+ Nylon membrane, one piece of nitrocellulose membrane and two pieces of 3 mm filter paper were hydrated in 10 x SSC for 5 minutes. The pre-wet 3mm

filter papers followed by the nitrocellulose and nylon membranes were placed onto the base of the dot-blot apparatus. The dot-blot apparatus was clamped together and the vacuum system set up as shown in Figure 2.7. Excess SSC was removed using a light vacuum. Each well was re-hydrated using 400 µl 10 x SSC, and then the SSC was removed by vacuum. The denatured PCR product was carefully loaded into the dot-blot wells and left to stand for 20 minutes. The liquid was then filtered through the membrane using a gentle vacuum until no excess liquid was visible in the wells. The wells were rinsed with 400 µl 10 x SSC and the membrane carefully removed from the blotter and immersed in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 5 minutes. The membrane was neutralised for 1 minute in 1.5 M NaCl, 0.5 M Tris HCl, 0.001 M EDTA, pH 7.0 and then allowed to dry for about 1 hour at room temperature. Once dry the DNA was permanently attached to the membrane by exposing it to 254 nm UV light (5000 µJoules cm^{-2}) for 30 seconds using the UV crosslinker (Spectronics Corporation).

DNA hybridisation (for dot and Southern blot)

The nylon membrane was re-hydrated in 2 x SSC for 5 minutes and placed into a 50 ml polypropylene tube with the DNA bound side facing inwards. Prehybridization mix (6 x SSC, 5 x Denhardt's solution (Amresco), 0.01 M EDTA pH 8.0 and 0.1 mg ml^{-1} denatured salmon sperm DNA (Invitrogen)) was placed into the tube and incubated for 30 minutes at a temperature less than the melting temperature of the probe (Table 2.6).

Prehybridization mix was removed from the tube and the hybridization mix (6 x SSC, 5 x Denhardt's solution, 0.01 M EDTA pH 8.0, 0.1 mg ml^{-1} denatured salmon sperm DNA, and 0.5 ng $µl^{-1}$ denatured biotin labelled internal probe) was added. Hybridization was allowed to proceed overnight with constant rotation in a hybridization oven at a temperature less than the melting temperature of the probe (Table 2.6).

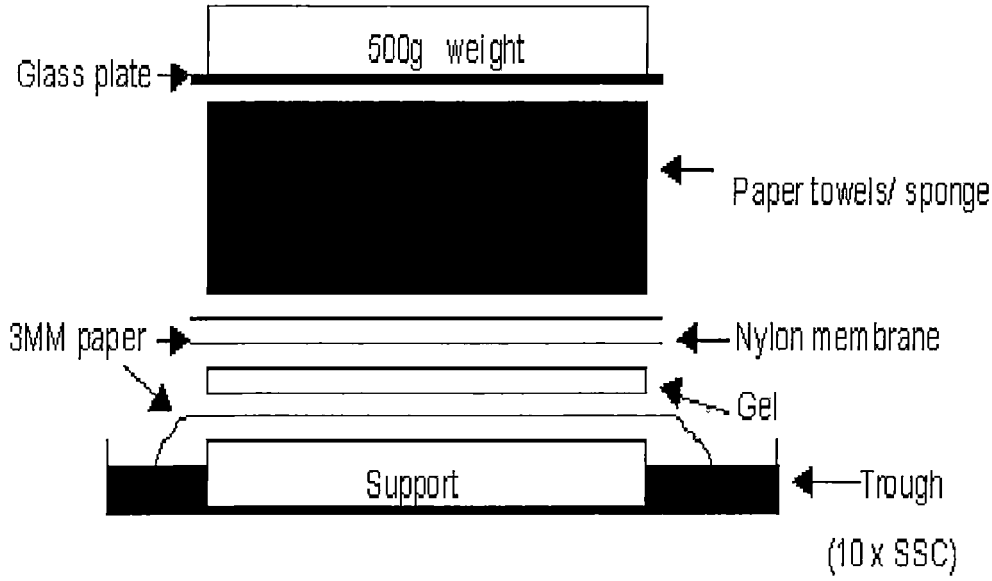


Figure 2.6: Diagram of Southern blot set-up.

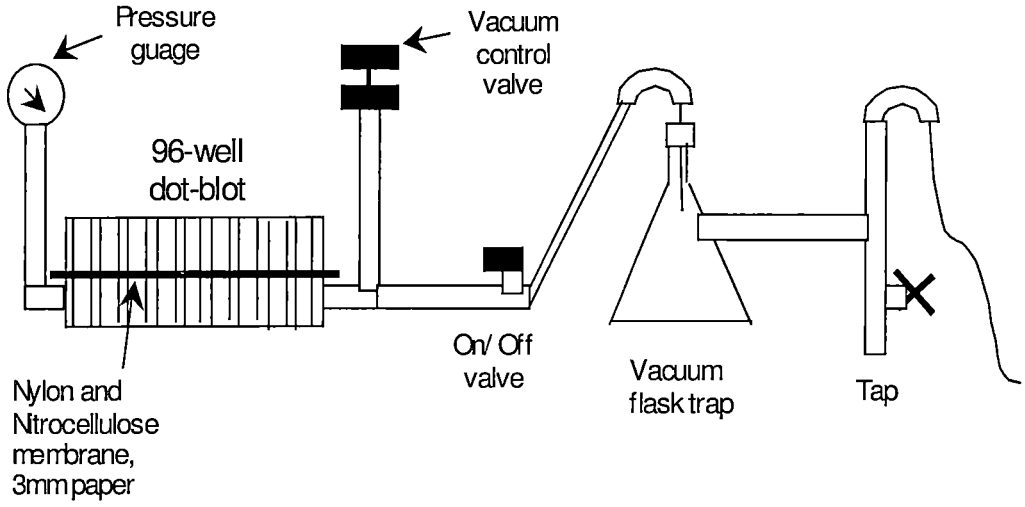


Figure 2.7: Diagram of Dot-blot set-up.

Table 2.6: Hybridization and post-hybridization temperatures trialed for dot and Southern blotting. The figures expressed in bolt type resulted in the best sensitivity without compromising the specificity.

Bacterium	Melting temperature of probe (nearest neighbour method)	Pre-hybridization and hybridization temperatures	Post-hybridization tempertures
A. salmonicida	56°C	48 50	40 45
L. garvieae	58°C	50 52 **54** 56	45 **50**
T. maritumum	56°C	48 50 **52** 54	45 **50**
Y. ruckeri	61°C	53 55 **57** 59	45 50 55 **60**

Post-hybridization wash

Hybridization mix was removed from the tube and the membrane was washed twice with 2 x SSC and 0.1% SDS at room temperature for 5 minutes. The membrane was washed twice in pre-heated 0.1 x SSC with 0.1% SDS for 20 minutes at the desired temperature (Table 2.6).

Detection of probe

The nylon membrane was placed in a flat-bottomed dish and washed for one minute in 0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl and incubated for 1h at 65°C in 0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl and 3% (w/v) bovine serum albumin (Fraction V).

The wash was removed from the membrane and streptavidin alkaline phosphatase (Promega) diluted to 1 mg ml^{-1} in 0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl was pipetted on top of the membrane. Conjugate binding was allowed to proceed at room temperature for 10 minutes with gentle agitation, occasionally pipetting the solution over the membrane.

The conjugate solution was removed and the membrane was washed twice with at least 200 ml of 0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl with gentle agitation for 15 minutes each time. The membrane was then washed in 0.1 M Tris-HCl (pH 9.5), 0.15 M NaCl, 50 mM $MgCl_2$ for 10 minutes at room temperature.

Visualisation

The nylon membrane was placed in a clip-sealed bag. A volume of 50 µl 50 mM nitro blue tetrazolium (Promega) was added to 7.5 ml of 0.1 M Tris-HCl (pH 9.5), 0.15 M NaCl, 50 mM $MgCl_2$, followed by 25 µl of 50 mM 5-bromo-4-chloro-3-indoxyl-phosphate (Promega). This solution was added to the clip-sealed bag and incubated at room temperature in the dark or in low light for 30 - 60 minutes. The membrane was washed in 1.5M NaCl, 0.5M Tris HCl, 0.001M EDTA (pH 7.0) to stop the colour development, rinsed in water and then air-dried.

RESULTS:

The Southern and dot blot techniques produced positive results for each bacteria except *A. salmonicida*. For these systems a sensitivity of at least 100 fg was achieved. With the *L. garvieae* and *T. maritimum* systems a hybridization temperature of 4°C below the melting temperature of the probe and a post-hybridization temperature of 50°C gave specific results. The specificity was tested by running a PCR for *A. salmonicida*, *Y. ruckeri*, *T. maritimum* and *L. garvieae* using 1 pg of target DNA as template, running the resulting amplicons on a single gel and using one of the internal probes to detect the appropriate target DNA. However when using the *Y. ruckeri* internal probe a post-hybridization wash temperature of less-than or equal–to the hybridization temperature, no matter how high the hybridization temperature was, resulted in cross-reaction with *A. salmoncida*. Increasing the post-hybridization wash temperature to 60°C prevented this cross-reaction but the sensitivity of hybridization was decreased to 1 pg as a direct effect of this temperature change, see Figure 2.8.

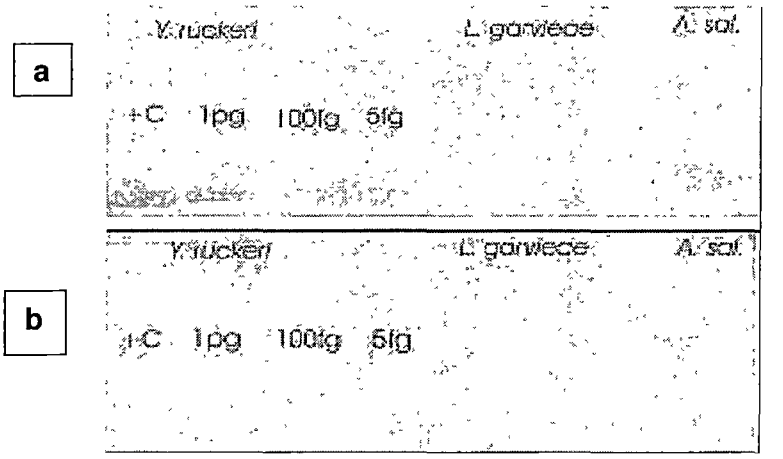


Figure 2.8: Sensitivity and specificity of *Yersinia ruckeri* Southern blot. [a] Hybridization and post-hybridization wash temperature 57°C. [b] Hybridization temperature 57°C and post-hybridization wash temperature 60°C.

DISCUSSION:

Proof of concept was successfully achieved for all systems except *A. salmonicida*, with optimum hybridization temperatures established for the internal probes. Repeated attempts using the hybridization protocol at differing hybridization temperatures, post-hybridization wash temperatures and internal probe concentrations failed to result in any trace of a positive result for *A. salmonicida*. However, the positive *A. salmonicida* band that occurred using the *Y. ruckeri* internal probe proved that the hybridization procedure should work. A fresh batch of the internal probe was obtained from the manufacturer, but repeat testing still failed to produce positive results. No more follow-up of this problem occurred at this stage. When developing the EHA system (Chapter 5) a new *A. salmonicida* probe was purchased from a different supplier and hybridization in the microtitre tray well was successful on the first attempt. The cross-reaction of the *Y. ruckeri* probe and the *A. salmonicida* DNA was explained by checking the base sequences of the *Y. ruckeri* and *A. salmonicida* internal probes and it was revealed that there is only a one base difference between these two probes.

The same sensitivity and specificity was achieved for Southern and dot blotting. The Southern blot technique was the simplest blotting method to

perform with capillary blotting occurring overnight. The dot-blot technique required optimization of membrane wetting techniques and the vacuum system to produce clearly readable dots that did not spread too much or form 'donut' shapes instead of entire dots. However once optimised, the dot blot technique was the technique of choice as it could be performed in a much shorter time and has the capacity to run 96 samples simultaneously.

2.3. DEVELOPMENT OF A SELECTIVE-ENRICHMENT MEDIUM FOR *Aeromonas salmonicida* biovar acheron

MATERIALS AND METHODS:

Minimum Inhibitory Concentration (MIC) assays

To develop a new selective-enrichment medium for *A. salmonicida* biovar acheron, information collected when developing the current selective-enrichment media HK (T. Wilson and J. Carson, proprietary formulations, Cooperative Research Centre for Aquaculture Ltd, Sydney) and new Minimum Inhibitory Concentration (MIC) data was used to determine likely useful antibacterials, as listed in Table 2.7. Bacteria used in the development of the medium were those which showed a similar resistance pattern to *A. salmonicida* during the development of the current media and those found to be resistant to the current media during fish trials, see Table 2.8.

The sensitivity data required for the development of the media was obtained using a broth dilution MIC method, based on the National Committee for Clinical Laboratory Standards (NCCLS) method (Sahm and Washington II 1991). While concentrations varied depending on the antimicrobial, a typical dilution method is demonstrated using an antimicrobial of 1000 $\mu g\ ml^{-1}$ stock concentration to produce a 25 $\mu g\ ml^{-1}$ concentration in the first row of the Microtitre tray. The antimicrobial was diluted 1:20 in basal medium (Cooperative Research Centre for Aquaculture Ltd, proprietary formulation) to give a concentration of 50 $\mu g\ ml^{-1}$. Doubling dilutions were prepared as tabled:

Column Number	Initial Concentration ($\mu g\ ml^{-1}$)	Final Concentration ($\mu g\ ml^{-1}$)
1	50.0	25.0
2	25.0	12.5
3	12.5	6.25
4	6.25	3.13
5	3.13	1.56
6	1.56	0.78
7	0.78	0.39
8	0.39	0.20
9	0.20	0.10
10	0.10	0.05
11	0.05	0.02
12	Control	Control

Young actively growing cultures of the bacterium in the early stage of logarithmic growth were used for the assays (Baker, *et al.* 1983). Suspensions of the test organism were prepared in the basal medium to approximate a McFarland 0.5 standard suspension, using a Hach Turbidimeter Model 2100P (Hach, Colorado, USA). This suspension was then diluted 1:100 in basal medium.

Antimicrobial dilutions were made in racked Micronic tubes using 250 µl volumes. Fifty microlitres of each dilution and 50 µl of the test organism suspension were transferred into a sterile microtitre tray using a multichannel pipette. The microtitre tray was sealed using self-adhesive plastic film sealer (ICN, CA, USA). The plates were incubated at 25°C until growth (turbidity) could be clearly seen in the control wells. Growth or no-growth was recorded for each organism. Light haziness in the endpoint well is normally not considered significant for chemotherapy of bacterial infections (Turnidge and Stockman 1991). However, for developing a selective medium any growth was considered significant since it would indicate a potential for growth and hence compromise performance of the medium. Therefore, the endpoint (MIC) was taken as the lowest concentration showing no growth visible to the naked eye (Waterworth 1978). The product limiting concentration (PLC) was the last well with a turbidity equal to the growth in the control well. Therefore, the PLC would have a concentration of antimicrobial agent less than the MIC. The MIC and PLC were recorded as the concentration of antimicrobial in $\mu g\ ml^{-1}$.

Table 2.7: Antibacterial agents used in the development of a selective medium for *A. salmonicida* biovar acheron.

Antibacterial agents	
Antibacterial H	Alexidine
Antibacterial K	Amoxicillin
Antibacterial C1	Ampicillin
Antibacterial C2	cis/trans Citral
Antibacterial C3	Citronellal
Lavendulol	Eugenol
Lonone	Colistin methane sulphonate
Nerol	Doxycycline
Palmarosa oil	Geraniol
β-Pinen	Geranium oil
R+ -Limonene	Polymixin B
α-Terpinene	Phosphomycin
α-Terpineole	Piromidic acid
Ofloxacin	Sulphaquinonaline
8-azaguanine	Sulphaquinoxaline
Thymol	Trimethoprim

Table 2.8: Bacteria used for developing the selective-enrichment medium for *A. salmonicida* biovar acheron.

Bacteria tested	
(B7) *Pseudomonas* sp.	(B11) *Aeromonas* sp.
(B56) *Pseudomonas* sp.	(D32) *Vibrio* sp.
(B91) *Pseudomonas* sp.	(D33) *Vibrio* sp.
(B127) *Pseudomonas* sp.	(B109) *Flavobacterium* sp.
(B128) *Pseudomonas* sp.	(M128) *Flavobacterium* sp.
(M146) *Pseudomonas* sp.	(B9) *Shewanella* sp.
(G49) *Pseudomonas* sp.	(B6) *Enterobacter* sp.
(FT1) *Pseudomonas* sp.	(M147) *Enterobacter* sp.
(W57) *Pseudomonas* sp.	**Proteus* sp.
(W43) *Aeromonas* sp.	**A. hydrophila*

All test bacteria were isolated from healthy fish as part of Co-operative Research Center (CRC) for Aquaculture study.

*Bacteria isolated from previous versions of *A. salmonicida* selective-media.

Chequerboard MIC assays

To check for antibacterial combined effects such as antagonism or synergy, chequerboard MIC assays were conducted with those antibacterials that showed potential as ingredients for a selective-enrichment medium. The method is demonstrated with two arbitrary antibacterial agents, antibacterial H and antibacterial K.

A 100000 µg ml^{-1} stock solution of antibacterial H was prepared in RO water (<2 µS). An aliquot of this stock was diluted 1:16 in the basal medium to give a concentration of 6240 µg ml^{-1}. A 1000 µg ml^{-1} stock concentration of antibacterial K was prepared in RO water. An aliquot of this stock was diluted 1:80 in the basal medium to give a concentration of 12.5 µg ml^{-1}. From these dilutions, 250 µl of the following doubling dilutions were prepared in Micronic tubes as tabled:

Antibacterial H:

Column Number	Initial Concentration (µg ml^{-1})	Final Concentration (µg ml^{-1})
1	6240	1560
2	3120	780.0
3	1560	390.0
4	780.0	195.0
5	390.0	97.50
6	195.0	48.75
7	97.50	24.38
8	48.75	12.19
9	nothing added	nothing added
10 (Row A)	(H)6240	(A)3120
11 (Row A)	(K)12.50	(B)6.250

Antibacterial K:

Row Number	Initial Concentration (µg ml^{-1})	Final Concentration (µg ml^{-1})
A	12.50	3.125
B	6.250	1.563
C	3.125	0.781
D	1.563	0.391
E	0.781	0.195
F	0.391	0.098
G	0.195	0.049
H	0.098	0.024

Fifty microlitres of each antimicrobial dilution was transferred to a microtitre tray as shown in Figure 2.9.

Young actively growing cultures of *A. salmonicida* biovar acheron, in the early stage of logarithmic growth were used for the assays (Baker, *et al.* 1983). Suspensions of the test organism were prepared in the basal medium to approximate a McFarland 0.5 standard suspension. This suspension was prepared in a Hach Turbidimeter by adding cells to 15 ml PBS (0.1 M, pH 7.2) until a reading of 90 NTU was achieved (Wilson 1996). The suspension was then diluted 1:100 in the basal medium, and 50 µl was added to each well of the microtitre tray. The microtitre tray was sealed using self adhesive plastic film (ICN), and incubated at 25°C for 3 days, after which time growth or no-growth was recorded for each well (turbidity in well).

		Concentrate ---------- H ----------→ dilute									H	K	Control
Conc.		1	2	3	4	5	6	7	8	9	10	11	12
↓	A										Conc.	Conc.	
	B										↓	↓	
K	C										↓	↓	
↓	D										↓	↓	
	E										↓	↓	
	F										↓	↓	
	G										↓	↓	
Dilute	H										Dil.	Dil.	

Figure 2.9: Chequerboard Microtitre tray layout.

Most Probable Number (MPN) assays

In order to quantify any inhibitory effect that the antibacterial agents contained in the selective medium had on the growth of *A. salmonicida*, an MPN comparison was made between growth in the base medium and growth in the selective medium. The MPN method is a means for estimating, without any direct count, the density of organisms in a liquid (Cochran 1950). The MPN was performed in a microtitre tray following the method of Wilson (1996), summarised as follows:

Prototype *A. salmonicida* biovar acheron medium (SEC+) and the modified BHI base media (SEC-) were each dispensed as 200 µl volumes into half of a

microtitre tray using a multistepper pipette. *A. salmonicida* biovar acheron was grown in pure culture and from this a McFarland standard 0.5 suspension was prepared in the Hach Turbidimeter and the suspension then diluted by 10^{-5}. Two samples were taken directly from the 10^{-5} suspension, the second sample being half the volume of the first. The 10^{-5} suspension was diluted ten fold, twice, by the addition of a volume of uninoculated medium, a system of dilution by addition, and two samples taken at each step. The system of inoculation is summarised in Figure 2.10. Five rows of the microtitre tray were used for replicates. Additions as described were made for the selective medium (SEC+) and the corresponding basal medium (SEC-). The plates were sealed using microtitre tray self-adhesive tape.

	From 10^{-5} ↓		From 10^{-6} ↓		From 10^{-7} ↓		From 10^{-5} ↓		From 10^{-6} ↓		From 10^{-7} ↓	
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	10µl	5µl	10µl	5µl	10µl	5µl	10µl	5µl	10µl	5µl	10µl	5µl
	SEC+						SEC-					

Figure 2.10: Row A of a Microtitre tray depicting volumes used for the MPN dilution series.

The microtitre tray was incubated at 25°C and read at 24 hour intervals. Turbidity in the wells indicated growth and could be seen with the aid of a plate reading mirror. When the same results were read for 2 consecutive days the final results were recorded.

Statistical Analysis of MPN data

The Most Probable Number method (McCrady 1915) was used to estimate the density of bacteria in the standard suspension and thus the level of inhibition by the selective agents. The method assumes:

(1) each sample exhibits growth if it contains at least one organism;

(2) the distribution of organisms is random without aggregation of any kind.

The MPN analysis was generated for the number of positive wells obtained for selective and non-selective media using an effective and flexible DOS based

computer program by González (1996). Student's t tests were then performed on the data to test for significant difference of estimates (Koopmans 1987).

Evaluation of the capacity of *A. salmonicida* biovar acheron to grow in the selective medium with competition

No selective media are perfect, and some non-target bacteria will always tolerate the antibacterial agents and grow in the media. In a volume of media there is only a limited supply of nutrients available for bacterial growth, therefore it is possible that a fast growing contaminant could out compete the target bacterium, inhibiting the ability of the target bacterium to multiply to a detectable level. Growth rates were assessed by placing the target bacterium and a resistant organism together in the selective medium and monitoring the growth of each bacterium.

Two resistant isolates, *Pseudomonas* sp. (B127) and a mucoid *Pseudomonas* sp. (M146) were used for the study. Both the target organism and the resistant organism were prepared to a density of about McFarland 0.5 and diluted 1:200 in the selective enrichment medium. At 24 hours, 3 days and 5 day, samples were taken from the medium and the Miles and Misra plate count method (Miles, *et al.* 1938) was used to count the number of each bacterium present. A numerical value for the ability of the target bacterium to grow in competition was calculated using the formula of Rhodes, *et al.* (1985):

$$\text{Enrichment Ratio (ER)} = \frac{\text{Viable conc. of target pathogen after enrichment}}{\text{Viable concentration of competitor after enrichment}}$$

$$\text{Initial Ratio (IR)} = \frac{\text{Initial viable concentration of target pathogen}}{\text{Initial viable concentration of competitor}}$$

$$\text{Enrichment Factor (EF)} = \frac{\text{ER}}{\text{IR}}$$

$$\text{Enrichment Index (EI)} = \log_{10}\text{EF}$$

The EI is a measure of the change in relative concentration of the target organism compared with the resistant organism over the enrichment period. The larger the EI the greater the enrichment of the target pathogen compared with the competing bacterium.

Evaluation of the specificity of the medium with 400 normal fish flora isolates

The final version of the selective-enrichment medium was tested for inhibition of a library of 400 normal fish flora isolates. These bacteria are held at the Department of Primary Industry Water and Environment (DPIWE) and were collected during the development of selective-media for *Y. ruckeri*, *L. garvieae*, *T. maritimum* and the first version of the medium for *A. salmonicida* (T. Wilson and J. Carson, proprietary formulations, Cooperative Research Centre for Aquaculture Ltd, Sydney).

The normal flora were revived from Microbank beads (Pro-Lab Diagnostics, Ontario, Canada) frozen at –80°C, and cultured on brain heart infusion agar (Oxoid, UK). Growth of the normal fish flora in the selective-enrichment medium was tested by adding approximately 7.5×10^5 CFU ml^{-1} of each bacterium (1:200 dilution of a McFarland 0.5 standard as used for standard minimum MIC assays) to a 200 µl volume of media set up in microtitre trays. This cell suspension was prepared using a version of the calibrated dichotomous sensitivity (CDS) test created by Bell (1975) and modified by Wilson (1996). The microtitre trays were incubated at 25°C and evidence of growth of the bacteria, and therefore resistance to the medium, was determined visually by detecting turbidity in the microtitre tray wells as seen with the aid of the plate reading mirror. The wells were checked for turbidity over a 5 day period.

RESULTS:

Development of the selective-enrichment medium

The composition of the selective-enrichment medium is proprietary FRDC information and can not detailed in this thesis.

A. salmonicida biovar acheron showed good resistance to three antibacterials contained in previously developed *A. salmonicida* selective media (T. Wilson and J. Carson, proprietary formulations, Cooperative Research Centre for Aquaculture Ltd, Sydney). Chequerboard MIC assays were performed first between these anitbacterials to formulate a basal selective medium for the bacterium. There was no evidence of antagonists or synergistic effects between these antibacterials, and the calculated maximum combined concentrations were 200 μg ml^{-1} antibacterial H, 0.8 μg ml^{-1} antibacterial K and 20μg ml^{-1} antibacterial C1. Two new, potentially useful, antibacterial agents were isolated from those tested, these are given the names C2 and C3. Antibacterial C2 inhibited about 25% of the normal flora that were resistant to the basal selective medium, and antibacterial C3 inhibited the growth of *A. hydrophila*, see Table 2.9. The bacteria that show some sensitivity to an antibacterial have their results highlighted in the table.

Table 2.9: MIC results for antibacterial C2 and antibacterial C3 with normal flora resistant to basal selective medium.

Bacterium	Antibacterial C2		Antibacterial C3	
	MIC (μg ml^{-1})	PLC [b] (μg ml^{-1})	MIC (μg ml^{-1})	PLC [b] (μg ml^{-1})
A. salmonicida biovar acheron	2500	625	125	62.5
(B7) *Pseudomonas* sp.	**7.8**	**3.9**	125	62.5
(FT1) *Pseudomonas* sp.	500	250	125	62.5
(W57) *Pseudomonas* sp.	500	250	>500	>500
(M146) *Pseudomonas* sp.	500	500	>500	>500
(B127) *Pseudomonas* sp.	1000	500	>500	>500
(G49) *Pseudomonas* sp.	**125**	**12.5**	**1.56**	**0.78**
(W43) *Aeromonas* sp.	>500	>500	125	31.25
(B11) *Aeromonas* sp.	>500	>500	>500	>500
(B109) *Flavobacterium* sp.	2000	2000	Not tested	Not tested
(B9) *Shewanella* sp.	**31.25**	**15.6**	Not tested	Not tested
(B6) *Enterobacter* sp.	**62.5**	**31.25**	250	125
Proteus sp. AT96[a]	500	125	>500	>500
A. hydrophila AT97[a]	>500	>500	**31.25**	**15.63**

[a] Bacteria resistant to old versions of selective media, isolated during fish trials

[b] Product limiting concentration

Chequerboard MIC assays were performed to determine the optimum concentration for antibacterial C2 and antibacterial C3 in the medium and to check for antagonists or synergistic effects. To perform this assay the previously calculated optimum concentrations of antibacterial H and antibacterial K were added to the base medium and antibacterial C2 and antibacterial C3 were titrated as described previously. No antagonistic or synergistic effects were seen, and four possible combinations of antibacterial concentrations were determined for antibacterial C2 and antibacterial C3. These four combinations were tested with those bacteria listed in Table 2.7 and the most effective combinations tested for their inhibitory effect on the target *A. salmonicida* by MPN. One version of the medium had a higher inhibitory effect on the normal flora tested and this version was chosen as the potential selective medium for the *A. salmonicida* biovar acheron. The final medium formulation, named HK3C, was as follows:

Antibacterial H: 200 $\mu g\ ml^{-1}$
Antibacterial K: 0.8 $\mu g\ ml^{-1}$
Antibacterial C1: 20 $\mu g\ ml^{-1}$
Antibacterial C2: 100 $\mu g\ ml^{-1}$
Antibacterial C3: 65 $\mu g\ ml^{-1}$

The HK3C medium prevented the growth of 80% of the bacteria listed in Table 2.7. The medium did not suppress the three pseudomonads (W57, M146 and B127) or the *Aeromonas* sp. (B11). When tested with the library of 400 normal fish flora isolates the total specificity of this new medium was 97%.

The MPN estimates for the growth of *A. salmonicida* biovar acheron in HK3C (4397 cells ml^{-1}) and the base non-selective growth medium (4307 cells ml^{-1}) were not significantly different (95% confidence limits). This result was confirmed using Student's t test (Koopmans 1987) on the MPN estimates (P=0.05). Therefore the selective components of the medium had no measurable inhibitory effect on the bacterium.

Evaluation of the capacity of *A. salmonicida* biovar acheron to grow in the selective medium with competition

A. salmonicida biovar acheron was able to grow in the presence of either competitor *Pseudomonas* sp. (B127) and *Pseudomonas* sp. (M146). *A. salmonicida* had negative EI values at 24 hours (-0.85), 3 (-0.17) and 5 days (-1.8) when grown in competition with *Pseudomonas* sp. (B127), showing this pseudomonad out-competed *A. salmonicida*, however the latter was still able to increase cell density 1000x after three days incubation. In the presence of the mucoid *Pseudomonas* sp. (M146), positive EI values at 24 hours (1.17), 3 (1.29) and 5 days (0.42) showed that *A. salmonicida* out-competed this pseudomonad.

DISCUSSION:

The new biovar of *A. salmonicida* failed to grow adequately in the selective media that had been developed for the greenback flounder and goldfish ulcer biovars of *A. salmonicida* (Carson and Wilson 2001). It was therefore necessary to develop a new version of the medium to accommodate this new biovar. The biovar acheron showed good resistance to three core ingredients, H, K and C1 of the previously developed selective medium. Past studies showed these antibacterials inhibited the growth of about 93% of a library of 400 normal fish flora (Carson and Wilson 2001). Antibacterial C2 showed good potential for inhibiting some of the resistant *Pseudomonas* species and some Enterobacteriaceae. During a preliminary field trial using an earlier formulation of the *A. salmonicida* biovar acheron selective medium, a strain of *A. hydrophila* was isolated. Because of the known cross-reaction of the 16S PCR primer set with *A. hydrophila*, further MIC testing was conducted to target this organism. A concentration of 31.25 - 62.5 $\mu g\ ml^{-1}$ of antibacterial C3 was found to inhibit the growth of this strain of *A. hydrophila* without inhibiting *A. salmonicida* biovar acheron.

As no antagonistic or synergistic effects were found between any of the antibacterials in the HK3C medium, the concentrations used in the final

formulation were determined as the highest concentration that allowed good growth in the microtitre tray well.

Using MPN analysis, no inhibition of the growth of *A. salmonicida* biovar acheron was found with the final version of the medium. Therefore theoretically, if one viable *A. salmonicida* biovar acheron cell was inoculated into the selective medium it would have as much chance of multiplying to a detectable level as if it were placed in the non-selective growth medium. While the medium appears to support the growth of *A. salmonicida* biovar acheron in laboratory conditions, its performance may be compromised when inoculated with fish tissue and potentially resistant normal flora.

A high specificity of 97% was achieved for the HK3C medium, this indicates that very few non-target bacteria are able to tolerate the medium and potentially out-compete *A. salmonicida* biovar acheron. The selective-enrichment medium provides an opportunity for the selective growth of the target bacterium and increases the likelihood of its detection.

No one medium has the ability to allow the growth of the target species and totally suppress undesired bacteria (Stainer 1988). Therefore when used in a field situation, even using the selective-enrichment medium, it is likely that the *A. salmonicida* biovar acheron will be required to grow in competition with other bacteria. The competition studies performed here showed that *A. salmonicida* biovar acheron was able to grow alongside either of the fast growing resistant *Pseudomonas* species tested. Even under competitive conditions *A. salmonicida* biovar acheron should be able to reach a cell concentration easily detectable by PCR.

CHAPTER 3: Prevention of False-Positive PCR Reactions Using Isopsoralen Compound 10 (IP-10).

INTRODUCTION:

Due to the sensitivity of PCR, the technology is prone to false-positive results arising from amplicon carry-over (Fahle, *et al.* 1999; Vaneechoutte and Van Eldere 1997). The frequency of these erroneous results can be greatly reduced by following strict containment policies such as separate rooms for sample preparation and amplification, dedicated equipment, barrier pipette tips, etc. (Kwok and Higuchi 1989). Containment procedures are not always sufficient in preventing carry-over contamination (Espy, *et al.* 1993; Padua, *et al.* 1999) which is often caused by aerosols and pipetting PCR amplicons (Cimino, *et al.* 1990; Saksena, *et al.* 1991).

False positive results are not acceptable when PCR is being used for quarantine and surveillance purposes. For critical applications a reliable method that prevents the re-amplification of PCR products needs to be implemented. One approach is to incorporate the photochemical isopsoralen compound 10 (IP-10) into the PCR reaction mixture and then activate the agent with UV light after amplification (Cimino, *et al.* 1991). When IP-10 is subjected to UV light (300 to 400 nm) it forms cyclobutane adducts with pyrimidine nucleotides (Cimino, *et al.* 1991), preferably thymidine (Espy, *et al.* 1993), which prevents subsequent PCR amplification by stopping *Taq* polymerase from processing along the amplicon (Persing and Cimino 1993). Therefore IP-10 is more effective on sequences that have a high adenine-thymine ratio or sequences that are greater than 350 bp in size (Espy, *et al.* 1993; Persing and Cimino 1993). The efficiency of activation of IP-10 also depends on the UV source. Studies suggest that a photothermal reaction chamber (HRI-300, Simms Instruments, Palo Alto, California) operating at 5°C is required for activating IP-10 to its maximum effectiveness (Fahle, *et al.* 1999; Rys and Persing 1993).

The work described here assesses the effectiveness of using a standard 300 nm UV transilluminator instead of an expensive photothermal reaction chamber to activate IP-10. It also assesses the ability of IP-10 to prevent false-

positive results due to carry-over contamination of amplicons from the four target bacteria. The target amplicons have adenine-thymine ratios of 45-51% and are very short, ranging from 145 – 288 bp.

MATERIALS AND METHODS:

Test nucleic acids

Test DNA was extracted using the large-scale extraction technique and test RNA was extracted using an RNAAqueous^{TM}-4PCR (Ambion) extraction kit as described in Chapter 2.1.

16S rRNA amplicons

The properties of the 16S rRNA amplicons were as follows: *A. salmonicida* (261 bp, 45% A+T); *T. maritimum* (288 bp, 51% A+T); *L. garvieae* (145 bp, 54% A+T) and *Y. ruckeri* (247 bp, 46% A+T).

Isopsoralen (IP-10) concentrations

IP-10 (Cerus Corporation, Ca, USA) was added to the PCR master mix in the following concentrations: 15, 20, 30, 40, 50 and 100 μg ml^{-1}.

PCR technique

PCR reactions were performed using no IP-10 and IP-10 at each of the five concentrations specified. The PCR reaction mix contained 200 μM each of dNTPs, 1.375 mM $MgCl_2$ for *A. salmonicida* and 2 mM $MgCl_2$ in all other cases, 1 X PCR buffer (Invitrogen), 2 μM each of the two primers, 10% glycerol, IP-10, 0.5 units Platinum *Taq* DNA Polymerase (Invitrogen), 1 μl template DNA (40 ng μl^{-1} unless specified otherwise) and sufficient 18 Mohm water to bring the total reaction volume to 20 μl. The PCR cycling conditions used were those given in Table 2.6. A positive control and a negative control were included in each PCR run.

RT-PCR technique

After the optimum concentration of IP-10 was determined for the PCR protocols, IP-10 was tested at this concentration for RT-PCR. This concentration was too high for optimum RT-PCR therefore RT-PCR reactions were performed using no IP-10 and IP-10 at 10, 20 and 30 μg ml^{-1}. The RT-PCR reaction mix contained 200 μM each of dNTPs, 1.375 mM $MgSO_4$ for *A. salmonicida* and 2 mM $MgSO_4$ in all other cases, 1 X RT-PCR buffer (Invitrogen), 2 μM each of the two primers, 10% glycerol, IP-10, 0.2 μl Superscript™ One-Step RT-PCR with Platinum *Taq* (Invitrogen), 1 μl DNase treated template RNA (40 ng μl^{-1} unless specified otherwise) and sufficient 18 Mohm water to bring the total reaction volume to 20 μl. The RT-PCR cycling conditions used were those given in Table 2.6. A positive control, a negative control with template but no RT enzyme and a negative control with no template RNA were included in each RT-PCR run.

Electrophoresis

Unless stated otherwise all results were visualized by gel electrophoresis on a 2% (w/v) agarose gel containing 0.5 μg ml^{-1} ethidium bromide (Sigma-Aldrich) prepared in TAE buffer (Appendix A). The gels were run using a horizontal gel electrophoresis apparatus (Invitrogen) at 70 V for 40 minutes.

PCR product inactivation

After amplification the samples were cooled to 4°C, placed directly onto the transilluminator (Ultra-Lum, Ca, USA) screen and covered with a flexible ice brick cooled to 4°C. The samples were irradiated with UV (300 nm) for 15 minutes. In order to determine the effectiveness of amplicon inactivation by IP-10, PCR product was immediately re-amplified by PCR. To confirm amplification, the remaining PCR product was visualised by electrophoresis.

Efficiency of inactivation

A 1 μl sample of the IP-10 treated PCR product was used as a template for subsequent PCR performed with no IP-10 in the reaction mix. A second 1 μl sample was diluted 1:100 and used to create three 10-fold dilutions of the PCR

product. The resulting dilutions were: neat, 10^{-2}, 10^{-3}, 10^{-4} 10^{-5}. The presence or absence of amplified product was determined by electrophoresis followed by Southern blot hybridization as described in Chapter 2.

Effect of IP-10 on PCR sensitivity

Purified DNA, extracted using Method 2.1, was used to prepare dilutions for the sensitivity assays. The DNA was diluted to 10 ng μl^{-1}, 100 pg μl^{-1}, 500 fg μl^{-1}, and then to 5 fg μl^{-1} in 10-fold dilutions, and used as a template for PCR. The sensitivity endpoint was determined by electrophoresis.

Effect of IP-10 on RT-PCR sensitivity

Purified RNA, extracted using a RNAAqueous^{TM}-4PCR extraction kit (Ambion, Austin, Texas, USA) was used to prepare dilutions for the sensitivity assays. The RNA was diluted to 10 ng μl^{-1}, 100 pg μl^{-1}, 500 fg μl^{-1}, and then to 5 fg μl^{-1} in 10-fold dilutions, and used as a template for RT-PCR. The sensitivity endpoint was determined by electrophoresis.

Southern blot hybridization

Southern blot transfer was performed for the inactivation and sensitivity assays, as described in Chapter 2.2.

Effect of IP-10 on amplicon mass

PCR was run with and without 40 $\mu g\ ml^{-1}$ of IP-10 and the amplicons were visualised by electrophoresis using a high quality agarose gel for small fragments (Promega). The size of the amplicons was measured using a 100 bp ladder (Advanced Biotechnologies) and a 25 bp ladder (Invitrogen).

IP-10 activation using a Spectrolinker UV crosslinker

PCR was set up as detailed above with IP-10 added to the PCR reaction mix at a concentration of 40 $\mu g\ ml^{-1}$. After amplification, the samples were cooled to 4°C, a flexible ice brick previously cooled to 4°C was placed onto the base of the UV crosslinker (Spectronics Corporation) and the samples placed on top. The samples were then irradiated with UV (365 nm) using an energy setting of 8800 mJ cm^{-2} (an approximation of the energy output resulting after

15 minutes of irradiation with new UV tubes). In order to determine the effectiveness of amplicon inactivation, PCR product was immediately re-amplified by PCR. To confirm amplification, the remaining PCR product was assayed by electrophoresis.

RESULTS:

PCR amplicons from *A. salmonicida* and *T. maritimum* appeared to be completely inactivated at an IP-10 concentration of 50 μg ml^{-1} using a UV transilluminator at 300 nm. However, when used at this concentration IP-10 decreased the sensitivity of the PCR. Amplicons from *L. garvieae* were not completely inactivated even when the concentration of IP-10 was increased; with *Y. ruckeri* and *A. salmonicida* increasing the concentration of IP-10 to 100 μg ml^{-1} blocked PCR amplification (Figure 3.1, lanes 5 and 6).

The highest concentration of IP-10 that could be added to the PCR without adversely affecting the specificity (data not shown) or sensitivity was 40 μg ml^{-1}; at this concentration it was possible to detect 5 fg of DNA by gel electrophoresis and Southern blot hybridization for each of the four bacteria (Figure 3.2). However when neat PCR product inactivated with 40 μg ml^{-1} IP-10 was re-amplified, a weak positive band was seen by gel electrophoresis. Presuming that the neat PCR product contained approximately 6×10^9 μl^{-1} copies of the amplicon (HRI Research Inc. 1991), dilution of this product showed that 40 μg ml^{-1} IP-10 could completely inactivate at least 6×10^7 amplicons. This level of inactivation was also achieved using 30 μg ml^{-1} IP-10 for *T. maritimum* and *Y. ruckeri*. Figure 3.3 shows the re-amplification assay for *Y. ruckeri* determined by gel electrophoresis and confirmed by Southern blot; the same results were obtained for the other bacteria (figures not shown). To show that this inactivation was not due to UV alone, *Y. ruckeri* PCR products not containing IP-10 were subjected to UV and re-amplified by PCR. The resulting PCR products are shown in Figure 3.4.

Effective inactivation using 40 μg ml^{-1} IP-10 was only achieved when the PCR products were cooled during UV activation using a flexible ice brick cooled to 4°C. It was not possible to measure the temperature of the PCR product

during UV irradiation but when the ice brick was omitted, IP-10 inactivation was not as efficient (Figure 3.5).

The binding of IP-10 onto amplicons increased their mass. With increasing concentrations of IP-10, the amplicons migrated more slowly through the gel, as shown in Figure 3.6. The increase in mass of the amplicons varied between the bacteria and the extent of the mass increase is given in detail in Table 3.1.

During sensitivity assays it was observed that 40 $\mu g\ ml^{-1}$ IP-10 decreased the sensitivity of the RT-PCR protocols by a factor of at least 100. Increasing the concentration of the co-solvent glycerol in the reaction mix did not improve sensitivity. The sensitivity of the RT-PCR protocols increased with decreasing concentrations of IP-10, with no sensitivity decrease detected when using 15 $\mu g\ ml^{-1}$ or less IP-10. Efficiency of inactivation experiments showed that 15 $\mu g\ ml^{-1}$ IP-10 was relatively ineffective in inactivating RT-PCR amplicons: a maximum of $6 \times 10^2\ \mu l^{-1}$ amplicons were inactivated for *L. garvieae* and $6 \times 10^3\ \mu l^{-1}$ amplicons inactivated for the other three bacteria.

Studies into the efficiency of amplicon inactivation using the UV crosslinker showed promising results. Assays conducted using 40 $\mu g\ ml^{-1}$ IP-10 showed this method of applying UV to be as effective as using the UV transilluminator, and probably better. Using 8800 $mJ\ cm^{-2}$ UV no bands were produced when the neat PCR products of *A. salmonicida*, *T. maritimum* and *Y. ruckeri* were re-amplified and only a weak positive band was seen by gel electrophoresis when neat PCR product from *L. garvieae* was re-amplified, but no band was seen from re-amplification of any of the diluted PCR product.

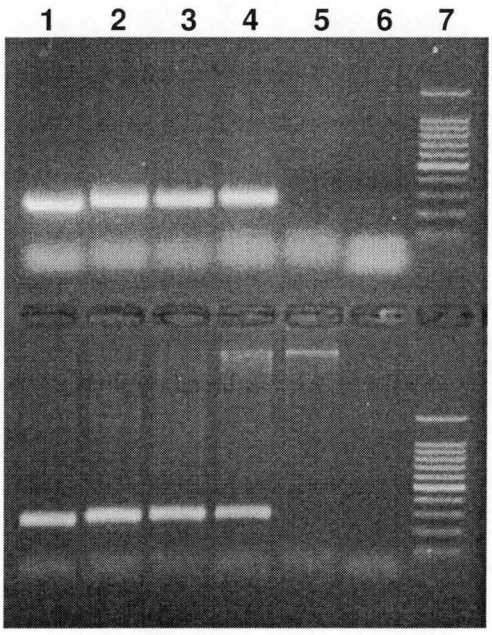


Figure 3.1: Effect of IP-10 on PCR amplicon mass with increasing concentrations of IP-10. Top row, *Yersinia ruckeri*: lane 1, amplicons with no IP-10; lane 2, amplicons with 30 µg ml^{-1} IP-10; lane 3, amplicons with 40 µg ml^{-1} IP-10; lane 4, amplicons with 50 µg ml^{-1} IP-10; lane 5, amplicons with 100 µg ml^{-1} IP-10; lane 6, negative control; lane 7, Advanced Biotechnologies 100 bp ladder. Bottom row, *Aeromonas salmonicida*: lane descriptions as for *Y. ruckeri*.

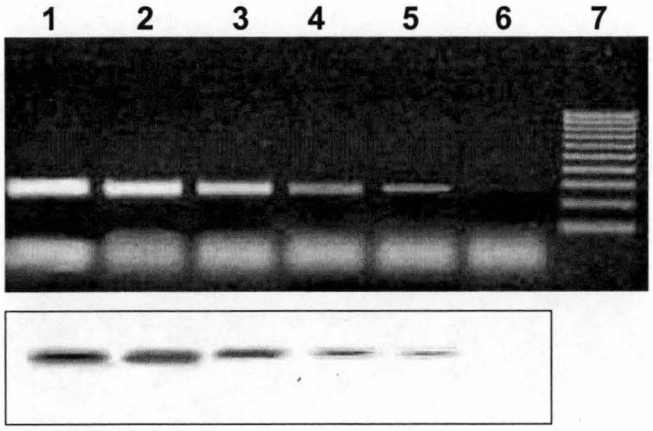


Figure 3.2: Sensitivity assay for *Yersinia ruckeri* with 40 µg ml^{-1} IP-10. Agarose gel electrophoresis and corresponding Southern blot. Lane 1, 10 ng $µl^{-1}$ DNA; lane 2, 100 pg $µl^{-1}$ DNA; lanes 3 to 5, decimal dilutions from 500 fg $µl^{-1}$ to 5 fg $µl^{-1}$ DNA; lane 6, negative control and lane 7, Advanced Biotechnologies 100 bp ladder.

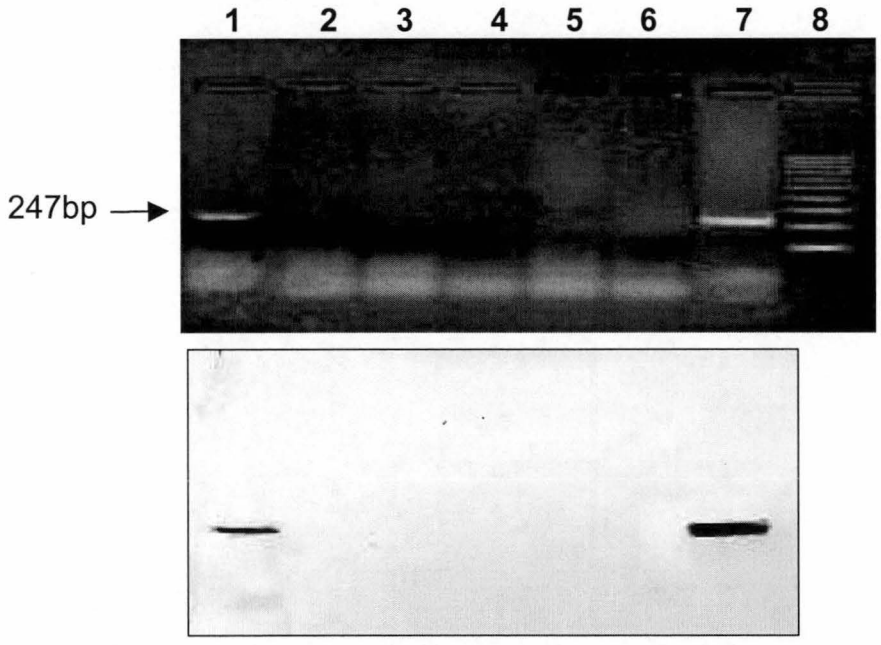


Figure 3.3: Re-amplification assay using *Yersinia ruckeri* as template, amplicon inactivated with 40 µg ml^{-1} IP-10. Agarose gel electrophoresis and corresponding Southern blot. Lane 1, approximately 6 x 10^9 amplicons; lanes 2 to 5, decimal dilutions from 6 x 10^7 to 6 x 10^4 amplicons; lane 6, negative control; lane 7, positive control; and lane 8, Advanced Biotechnologies 100 bp ladder.

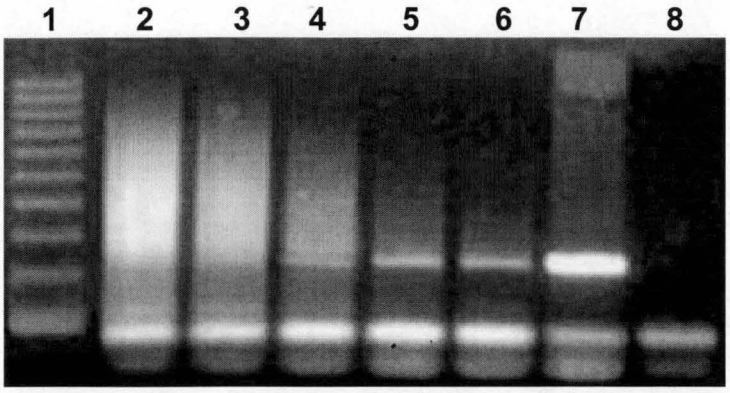


Figure 3.4: Re-amplification assay using *Yersinia ruckeri* as template, amplicon treated only with UV. Lane 1, Advanced Biotechnologies 100 bp ladder; lane 2, approximately 6 x 10^9 amplicons; lanes 3 to 6, decimal dilutions from 6 x 10^7 to 6 x 10^4 amplicons; lane 7, positive control; lane 8, negative control.

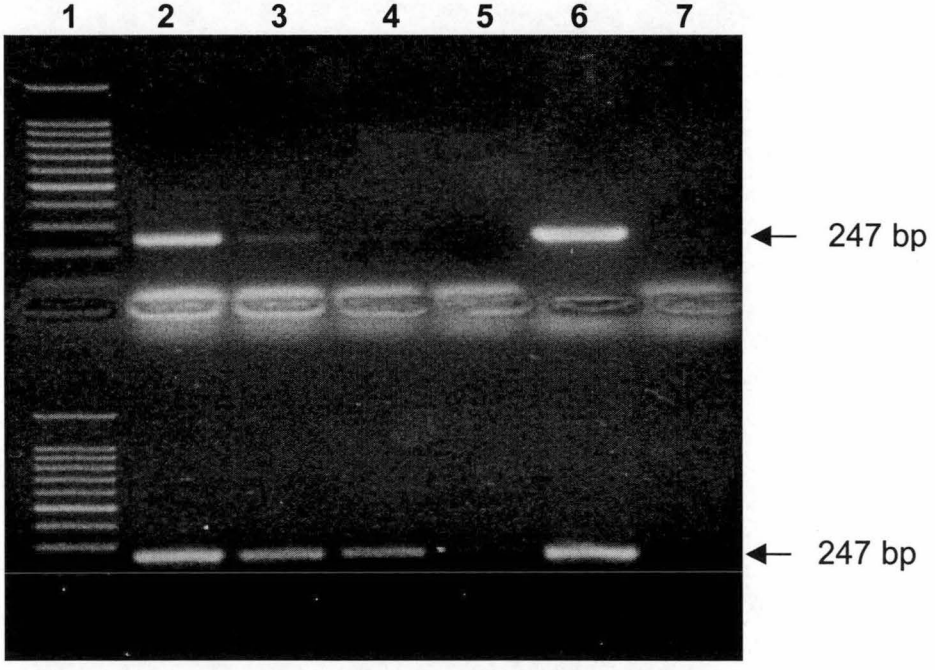


Figure 3.5: Effect of cooling on the efficiency of UV activation of *Yersinia ruckeri* PCR amplicons treated with 40 µg ml^{-1} IP-10. Top row, with cooling: lane 1, Advanced Biotechnologies 100 bp ladder; lane 2, approximately 6 x 10^9 amplicons; lanes 3 to 5, decimal dilutions of 6 x 10^7 to 6 x 10^5 amplicons; lane 6, positive control; lane 7, negative control. Bottom row, without cooling: lane descriptions as for top row.

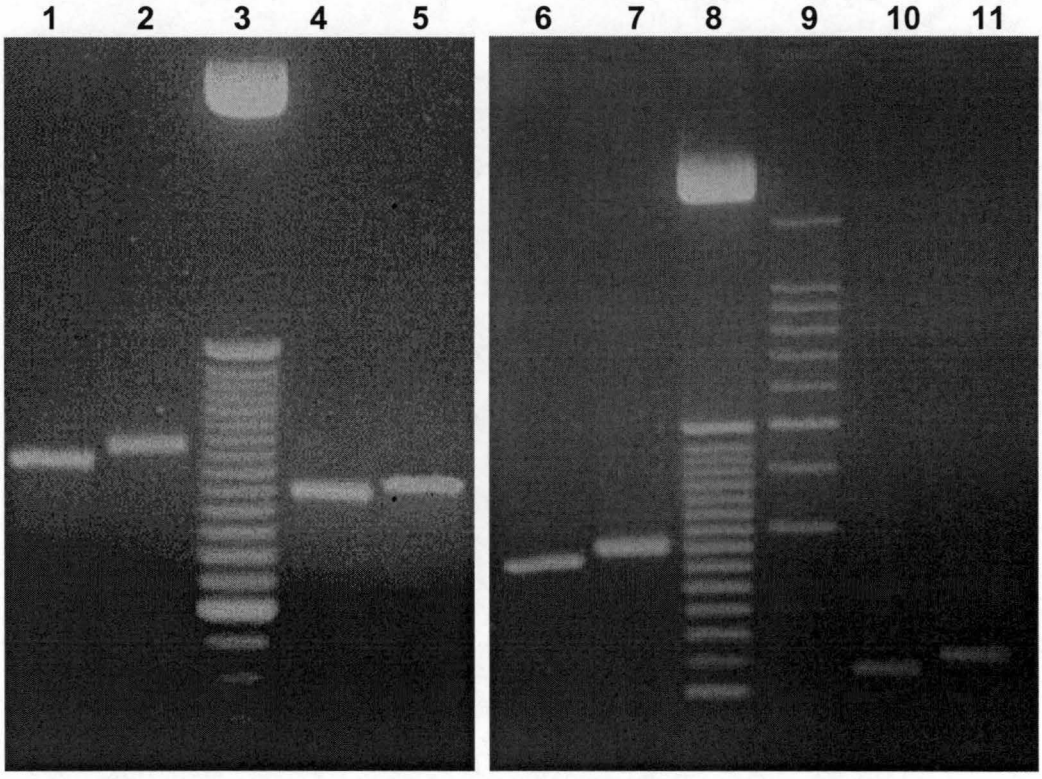


Figure 3.6: Effect of IP-10 at 40 µg ml^{-1} on amplicon mass. Lane 1, *Tenacibaculum maritimum* amplicon without IP-10; lane 2, *T. maritimum* amplicon with IP-10, measured size 320 bp; lane 3, Invitrogen 25 bp ladder; lane 4, *Yersinia ruckeri* amplicon without IP-10; lane 5, *Y. ruckeri* amplicon with IP-10, measured size 272 bp; lane 6, *Aeromonas salmonicida* amplicon without IP-10; lane 7, *A. salmonicida* amplicon, measured size 282 bp; lane 8, Invitrogen 25 bp ladder; lane 9, Advanced Biotechnologies 100 bp ladder; lane 10, *Lactococcus garvieae* amplicon without IP-10; lane 11, *L. garvieae* amplicon with IP-10, measured size 160 bp.

Table 3.1. Properties of PCR amplicons with and without IP-10.

Amplicon source	A+T ratio	Expected amplicon size	Mass with IP-10[a]	Mass increase[b]
A. salmonicida	45%	261 bp	282 bp	21 bp
L. garvieae	54%	145 bp	160 bp	15 bp
T. maritimum	51%	288 bp	320 bp	32 bp
Y. ruckeri	46%	247 bp	272 bp	25 bp

[a]Apparent mass of amplicon with 40 µg ml^{-1} IP-10

[b]Increase in mass due to IP-10

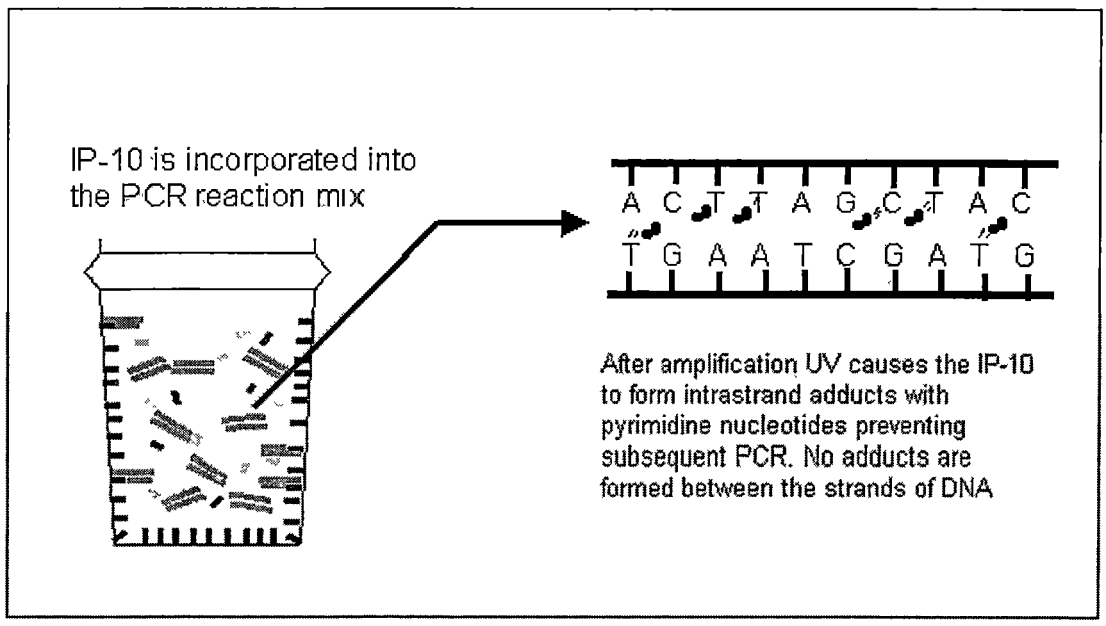


Figure 3.7: The formation of adducts between adjacent pyrimidine nucleotides by IP-10.

DISCUSSION:

Several PCR sterilization strategies have been developed with varying degrees of success. UV irradiation before PCR commences can be used to reduce the chance of false-positive PCR reactions (Ou, *et al.* 1991; Padua, *et al.* 1999). While this method can help inactivate clean low-level contamination that has already occurred, UV irradiation can compromise the integrity of primers and polymerases and may increase the chance of false-negative reactions. Uracil N-glycosylase (UNG) inactivation is reported to greatly reduce the chance of false-positive results due to amplicon carry-over (Burkardt 2000; Pang, *et al.* 1992). However the UNG inactivation method has disadvantages. Like IP-10, its efficiency is affected by the amount of thymine in the sequence and the amplicon length (Isaacs, *et al.* 1991). UNG is more expensive than IP-10 and care must be taken to prevent residual UNG activity from degrading amplified product prior to amplicon analysis (Rys and Persing 1993; Thornton, *et al.* 1992).

As UV irradiation and UNG pre-PCR contamination control methods have the potential to adversely affect PCR reagents prior to analysis, a better option for contamination control is to inactivate amplicons after amplification. Methods for post-PCR amplicon inactivation are based on UV activation of either psoralens or isopsoralens. Psoralens and isopsoralens are furocoumarins, planar, tricyclic compounds that can intercalate between base pairs of nucleic acids. Ultraviolet light activates these compounds causing them to form adducts between the nucleic acids of a PCR amplicon preventing *Taq* polymerase from processing along the amplicon, so stopping PCR amplification. Psoralens inactivate in a similar way to isopsoralens and are equally effective (Jinno, *et al.* 1990). However, during UV activation psoralens can form adducts between strands of double stranded DNA locking the strands together and therefore preventing the subsequent use of hybridization probes. Isopsoralens only form adducts between adjacent pyrimidine nucleotides when activated by UV light, interstrand adducts are not formed, therefore hybridization reactions are free to occur (Persing and Cimino 1993), see Figure 3.7.

In this study using isopsoralen, a standard 300 nm UV transilluminator proved to be an effective way of activating IP-10 and therefore inactivation of

PCR amplicons, significantly decreasing the risk of false-positive results occurring in subsequent PCR assays. While the definition of effective inactivation depends on the requirements of individual laboratories, generally inactivation of 10^5 amplicons is a minimum requirement (HRI Research Inc. 1991). De la Viuda, *et al.* (1996) and Espy, *et al.* (1993) successfully inactivated 10^5 amplicons of *Borrelia burgdorferi* (156 bp) and human papillomavirus type 16 (180 bp) respectively using 100 μg ml^{-1} IP-10. Using the definition of 10^5 amplicon inactivation the protocol described in this report is clearly effective in preventing the re-amplification of amplicons to a high level. In real terms, inactivation of 10^5 amplicons would decrease the chance of contamination due to aerosols (Cimino, *et al.* 1990). Therefore the inactivation of at least 6×10^7 amplicons reported here further increases the level of protection achieved with IP-10.

Complete amplicon inactivation requires IP-10 to create at least one adduct per strand of DNA. In practice, to ensure an adequate level of binding, Poisson statistics require a minimum average of 20 effective adducts per strand. Since isopsoralens can create 1 adduct per 11 to 100 nucleotides (Persing and Cimino 1993) depending on the amount of thymine and the UV conditions, complete inactivation requires amplicons to be at least 300-350 bp in length or have a strong bias towards pyrimidine nucleotides. It is not surprising then that complete inactivation of PCR amplicons could not be achieved with the short amplicons used in this study.

With all four bacterial species, carry-over of 6×10^9 IP-10 treated amplicons due to spillage or inadequate technique could contain enough functional DNA to cause a false-positive result in a new PCR reaction. While this amount of carry-over is unlikely, the possibility highlights the importance of using strict containment procedures to complement the use of IP-10.

Amplicons treated with IP-10 appeared to have an increased mass compared with amplicons not containing IP-10, as determined by gel electrophoresis. This increase in mass occurs as IP-10 binds to pyrimidine nucleotides, increasing the apparent size of the amplicon. Therefore an increase in the mass of the amplicon, is evidence of the formation of adducts and once an IP-10 protocol is established, mass of the amplicon can be used to indicate the on-going effectiveness of IP-10 inactivation (Cimino, *et al.* 1991). In

this study the larger amplicons of *T. maritimum*, *A. salmonicida* and *Y. ruckeri* had a greater increase in mass with the addition of IP-10 than the smaller *L. garvieae* amplicons, see Table 3.1 and Figure 3.6. This result suggests that the *L. garvieae* amplicons were not as effectively inactivated as the amplicons from the other three bacteria; this in-fact was the case. However despite *A. salmonicida* being a slightly larger amplicon than *Y. ruckeri*, the *Y. ruckeri* amplicons had a greater increase in mass after treatment suggesting more effective IP-10 binding, see Table 3.1 and Figure 3.6. This is probably due to the slightly higher thymine content of the *Y. ruckeri* amplicon, see Table 3.1. For the four species investigated, an increase in size of the amplicons appeared to match the efficiency of IP-10 inactivation.

As a UV transilluminator is designed for gel visualisation prolonged use would result in a decrease in output from the UV tubes resulting in decreased effectiveness of the UV activation. This problem can be avoided by using a UV-crosslinker to activate the IP-10. When using a crosslinker a desired UV output is administered over a period of time, as the UV tubes lose potency, the crosslinker adjusts the exposed time to compensate. Also, the UV crosslinker administers UV at a higher wavelength than the transiluminator, possibly resulting in an increase in the number of adducts created by the IP-10 (Persing and Cimino 1993).

It was only possible to add 15 μg ml^{-1} IP-10 to the RT-PCR protocol without causing a detrimental effect to the test sensitivity. As 15 μg ml^{-1} IP-10 did not inactivate the amplicons effectively it was not considered useful to add IP-10 to the RT-PCR protocols.

Incorporating IP-10 into the PCR protocol effectively prevented false-positive results without affecting the specificity or sensitivity of the PCR. Existing protocols were readily adapted to include IP-10 and satisfactory inactivation was achieved using a standard UV transilluminator making IP-10 accessible to laboratories that only contain basic PCR equipment.

CHAPTER 4: Rapid, High-Throughput Extraction of Bacterial Genomic DNA and RNA from Selective-Enrichment Culture Media.

INTRODUCTION:

Increasingly, PCR is being used for the detection of bacterial pathogens. While PCR has the capacity to detect a single genome, restrictions due to sample size, the presence of non-target DNA and PCR inhibitors mean that detection limits for direct PCR tend to lie between 10^4 and 10^5 cells g^{-1} sample (Sayer and Layton 1990; Swaminathan and Feng 1994; McIntosh, *et al.* 1996).

Greatly improved levels of detection have been achieved by coupling PCR with selective-enrichment culture (Giesendorf, *et al.* 1992; Hernandez, *et al.* 1995; Thisted Lambertz *et al.* 1996). Selective-enrichment culture allows for a large sample size, increasing the chance of the pathogen being present in the sample and, once in the media, the pathogen increases in number and competing microflora are suppressed (Fitter, *et al.* 1992; Swaminathan and Feng 1994; Witham, *et al.* 1996). The use of selective-enrichment-PCR (SEC-PCR) as a means of detecting covert infections in fish has been proposed (Carson 1997). The detection of covertly infected fish for health management, surveillance and quarantine using SEC-PCR requires the extraction of nucleic acids from large numbers of samples. Phenol phase separation and other nucleic acid extraction methods (Marmur 1961; Pitcher, *et al.* 1989; Boom, *et al.* 1990) are time consuming and cumbersome and commercial high-throughput systems are very expensive (typically over AU $3 per sample).

The research described here details the development of an inexpensive, high-throughput method of DNA and RNA extraction from selective-enrichment culture, suitable for use in PCR and RT-PCR. The method uses the lysing and nuclease-inactivating properties of guanidinium isothiocyanate (GuSCN) and the nucleic acid-binding properties of silica in the format of 96 well glass-microfibre filtration plates.

MATERIALS AND METHODS:

Evaluation of two 96-well extraction units

Evaluation of nucleic acid extraction systems was done on the basis of extracting DNA. Two 96-well vacuum manifold systems were trialed. A Multiscreen^{TM} FB filter plate (Millipore corp., MA, USA) and a vacuum manifold from Millipore, and the Whatman Polyfiltronics GF/B Uni-filter plate (Whatman, Rockland, MA, USA) and teflon coated vacuum manifold (Whatman). The systems were evaluated with respect to cost, ease of use, quality of the system, quality of the DNA extracted from selective-enrichment media, and other problems or factors as they occurred.

Extraction of genomic DNA and ribosomal RNA using Univac manifold and Whatman GF/B 96-well filter plates

Precautions for working with RNA.

When working with RNA all steps were taken to ensure that all equipment was free of RNases. Glassware was washed thoroughly and treated with 0.1% diethyl pyrocarbonate (DEPC) for 12 hours at 37 °C followed by autoclaving to degrade the DEPC. Sterile, disposable plasticware straight out of the packet was considered RNase-free. New, non-sterile plasticware was soaked in 0.1% DEPC for 2 hours and autoclaved before use. All solutions were made RNase-free by treatment with DEPC i.e. by adding 0.2 ml DEPC per 100 ml of solution, mixing vigorously, allowing the solution to stand overnight at room temperature and then autoclaving. Since DEPC is a suspected carcinogen, where possible the DEPC treatment was carried out in a fume hood. DEPC cannot efficiently inactivate solutions that contain Tris buffer, since Tris reacts with DEPC, therefore such solutions were prepared using DEPC-treated water then autoclaved before use.

Extraction reagents.

The reagents used for nucleic acid extraction were those specified by Boom, *et al.* (1990) with some modifications due to the required co-extraction of RNA. These are:

Lysis Buffer (L6). 30 g of guanidinium isothiocyanate (GuSCN) was dissolved by heating to about 60°C in 25 ml 0.1 M Tris-HCl, pH 6.4. The buffer was cooled and 5.5 ml of 0.2 M EDTA and 700 µl Triton X-100 (Sigma) were added. When extracting RNA 1% β-mercaptoethanol was added to the buffer just before use to inhibit RNase activity (DEPC was not added to the lysis buffer as it is a PCR inhibitor).

Wash Buffer (L2). 30 g GuSCN was dissolved by heating to about 60°C in 25 ml 0.1 M Tris-HCl, pH 6.4. When extracting RNA 1% β-mercaptoethanol was added to the buffer just before use.

Elution Buffer. DEPC treated 18Mohm water (10 mM Tris-HCl, pH 8.0 was used if extracting DNA only).

Sample source.

For nucleic acid extraction the bacteria were suspended in selective-enrichment media created for the individual bacteria: HK3C for *A. salmonicida*; CORT for *L. garvieae*; POSI for *T. maritimum* and POST for *Y. ruckeri* (T. Wilson and J. Carson, proprietary formulations, Cooperative Research Centre for Aquaculture Ltd, Sydney).

Extraction protocol.

Unless otherwise specified, all reagents were prepared with 18 Mohm water (DEPC treated when extracting RNA).

Pre-treatment for Gram positive bacteria. After inoculation and incubation, 1 ml samples of selective-enrichment medium were placed into 1.5 ml micro-titertubes (Quality Scientific Plastics, Porex Bio Products Group, Petaluma, CA, USA). Lysis was initiated by adding 100 µl of 40 mg ml^{-1} lysozyme in 0.1 M saline–EDTA to each racked sample, these were capped and gently agitated on a shaker table for

1 h at 37°C. For the extraction of DNA only, each sample was further treated by adding 2 µl of 50 mg ml^{-1} Proteinase K together with 100 µl of 10% (w/v) SDS and the tubes gently agitated on a shaker table for 30 min at 50°C. DNA and RNA samples were cooled at -20°C for 3 min, incubated at 95 – 100°C for 15 min and then cooled once more at -20°C for a further 3 min.

Extraction. Lysis, nucleic acid binding and elution were performed using a Polyfiltronics glass microfibre (type GF/B) 800 µl, 96 well Uni-filter plate (Whatman, Rockland, MA, USA), and a UniVac vacuum manifold (Whatman). Each well on the filter plate was wetted with water that contained 0.2% DEPC, and remained in contact for 1 hour before the wells were emptied by vacuum. Before use the L6 and L2 buffers were cooled to 4°C for at least 10 min. Each well on the filter plate was soaked with 100 µl of 10 mM Tris-HCl pH 6.4. After two minutes the wells were drained by applying a vacuum of about 20 kPa for about 1 min. A volume of 500 µl L6 buffer was added to each well followed by 200 µl of inoculated selective-enrichment broth. After 15 min the lysate was removed from the filter wells by applying a vacuum of about 13.5 kPa. A low vacuum pressure was essential at this step to prevent foaming of the L6 buffer around the drip directors underneath each well, a condition that could lead to sample cross-contamination. Flow rates between wells were balanced by sealing the plate with a flexible membrane (Polyfiltronics VacAssist, Whatman). The filter plate wells were washed twice with 100 µl of L2 buffer and then washed five times with 200 µl of 70% ethanol, and once with acetone at a vacuum pressure of 40 kPa. After the acetone wash the vacuum was maintained until there was no visible trace of solvents in the wells or on the drip directors underneath the wells. A 96 well microtitre plate to receive the eluted DNA was placed in the vacuum manifold and 35 µl of elution buffer, pre-heated to 90°C, was then added to each filter well. After 10 min, nucleic acids were eluted from the glass microfibre filter plate at a vacuum pressure of 40 kPa and a further 20 µl of elution buffer was placed in the wells. After 2 minutes the second volume of buffer was eluted and allowed to re-hydrate overnight at 4°C.

Positive controls.

DNA extracted using the large-scale extraction technique and RNA extracted using an RNAAqueous^{TM}-4PCR (Ambion) extraction kit as described in Chapter 2 were used for positive controls.

PCR of extracted DNA.

PCR reactions were performed in a 20 µl reaction volume containing 200 µM each of dNTPs, 2 mM $MgSO_4$ (1.375 mM for *A. salmonicida*), 1 X PCR buffer (Invitrogen), 10% (v/v) glycerol, 40 ng μl^{-1} isopsoralen (Cerus Corporation), 1.5 µg μl^{-1} Fraction V BSA (Sigma-Aldrich), 0.5 units Platinum Taq DNA Polymerase (Invitrogen), 2 µM each of the two primers, filter plate extracted DNA, pre-warmed to 25°C and enough 18 Mohm water to bring the reaction volume up-to 20 µl. PCR cycle conditions were those given in Table 2.5. A positive control and a negative control were included in each PCR. After amplification the samples were placed in a UV transilluminator and irradiated with 300 nm UV for 15 min to activate the isopsoralen and render the amplicons resistant to further amplification, as described in Chapter 3. Amplicons were visualised by gel electrophoresis on a 2% (w/v) agarose gel containing 0.5 µg ml^{-1} ethidium bromide (Sigma-Aldrich) prepared in TAE buffer (Appendix A) and their size measured using a 100 bp molecular weight marker (Advanced Biotechnologies). The gels were run using a horizontal gel electrophoresis apparatus (Invitrogen) at 70 V for 40 minutes.

RT-PCR of extracted RNA.

To achieve RNA free of DNA, the vacuum extracted nucleic acids were treated with DNase 1 enzyme (Promega), see the Development of RT-PCR protocols in Chapter 2. The DNase enzyme was activated by heating the nucleic acids to 37°C for 30 minutes, deactivated by heating to 75°C for 5 minutes and then cooled to 4°C before use. RT-PCR reactions contained 200 µM each of dNTPs, 1.375 mM $MgSO_4$ for *A. salmonicida* and 2 mM $MgSO_4$ in all other cases, 1 X RT-PCR buffer (Invitrogen), 2 µM of each primer, 0.2 µl Superscript^{TM} One-Step RT-PCR with Platinum *Taq* (Invitrogen), 1 µl DNase treated RNA and sufficient DEPC treated

18 Mohm water to bring the total reaction volume to 20 µl. Cycling conditions used were: cDNA from RNA at 50°C for 30 minutes followed by a 3 minute denaturation at 94°C, followed by 35 cycles consisting of denaturation at 94°C (30 sec), annealing at 65°C (30 sec), extension at 72°C (2 minutes), with a final extension at 72°C for 5 minutes. A positive control, a no-sample and a no-RT enzyme negative control were included in each RT-PCR. Amplicons were visualised by electrophoresis as described for PCR.

Optimisation of vacuum extraction.

The selective enrichment culture (SEC)-vacuum extraction protocol was optimised by varying the lysis and elution buffers and adding BSA to the extraction in an attempt to combat PCR inhibitors. The effectiveness of these variations was determined using inoculated SEC as follows: Selective-enrichment media were inoculated, and incubated overnight at 25°C. A faintly turbid suspension (approximately 1 x 10^5 cells ml^{-1}) was made from an overnight broth culture, and decimal dilutions were prepared using the selective-enrichment medium as diluent. Just prior to nucleic acid extraction viable counts of the suspensions were determined by the Miles and Misra method (Miles *et al.* 1938) using marine Shiehs medium (Appendix A) for *T. maritimum*, and Blood Agar Base No. 2 (Oxoid) supplemented with 7% (v/v) defibrinated sheep's blood (SBA) for the other bacteria; plates were incubated for 24-48 hours at 25°C. For *L. garvieae* and *Y. ruckeri* the total number of bacterial cells in the original suspension were determined by direct counts using dark field microscopy and a Helber counting chamber (Weber Scientific Int., Teddington, UK). Nucleic acids from each of the decimal dilutions were immediately extracted as described and the DNA or RNA amplified by PCR or RT-PCR to determine the endpoint of the titration.

The vacuum system was also optimised by checking for cross-contamination, speed and ease of high-throughput sampling. To check for cross-contamination selective-enrichment media seeded with *Y. ruckeri* were added to alternate wells of a glass microfibre filter plate. Vacuum extraction was performed, followed by PCR.

Optimisation of PCR and RT-PCR using vacuum extracted nucleic acids.

The vacuum extraction-(RT-)PCR protocol was optimised by adding 100 – 1500 ng μl^{-1} BSA to the PCR in an attempt to gain relief from PCR inhibitors and by varying the PCR template volume from 1 to 9 µl. The effectiveness of these variations was determined by performing decimal dilutions of extracted nucleic acids in 18 Mohm water, performing PCR or RT-PCR and determining the endpoint of the titration.

Effect of non-target bacteria on extraction sensitivity.

To determine the ability of the protocol to detect small amounts of the target bacterium among large amounts of non-target bacteria, a *Providencia* sp. was added to the selective-enrichment media just prior to extraction. A 24 hour culture of *Providencia* sp. on SBA was suspended in selective-enrichment medium to a density of about 1×10^{10} cells ml^{-1}. One hundred microlitres of this suspension was added to 900 µl of each decimal dilution containing the target bacterium, to give a final concentration of 1×10^{9} cells ml^{-1} of the *Providencia* sp.

Nucleic acids from each of the decimal dilutions was extracted using the protocol described, and the DNA or RNA amplified by PCR or RT-PCR.

RESULTS:

Evaluation of two 96-well vacuum extraction units

Two 96-well glass microfibre and vacuum manifold systems were evaluated. From the results in Table 4.1 it can be seen that the Whatman Polyfiltronics system is superior to the Millipore system, the main factors in this superiority were the volume capacity and the design of the micrifibre plate to allow a high sample volume and easy elution of small volumes of DNA, see Figure 4.1.

Table 4.1: Comparison of Whatman Polyfiltronics and Millipore 96-well vacuum filter systems.

Criterion	Whatman system	Millipore system
Cost vacuum manifold	AU$1,560	AU$995
Cost 96-well filter plates	AU$800/ 25 plates	AU$600/ 25 plates
Ease of use	Good	Good
Durability of system	Excellent	Good
Quality of eluted DNA	High	High
Sample volume	Maximum sample volume of 2ml	Maximum sample volume of 200 µl
Cross-talk between wells	Nucleic acids eluted through long drip directors which help form a drop with less elution volume and direct the drop into the correct well of the microtitre tray, see Figure 4.1.	Cross-contamination with small elution volumes
Problems encountered	Microfibre well blocked with cell debris if overloaded	Microfibre well blocked with cell debris if overloaded DNA sometimes difficult to elute, see Figure 4.1.
Other advantages	System has a vacuum assist to help even out the vacuum across the wells of the plate	

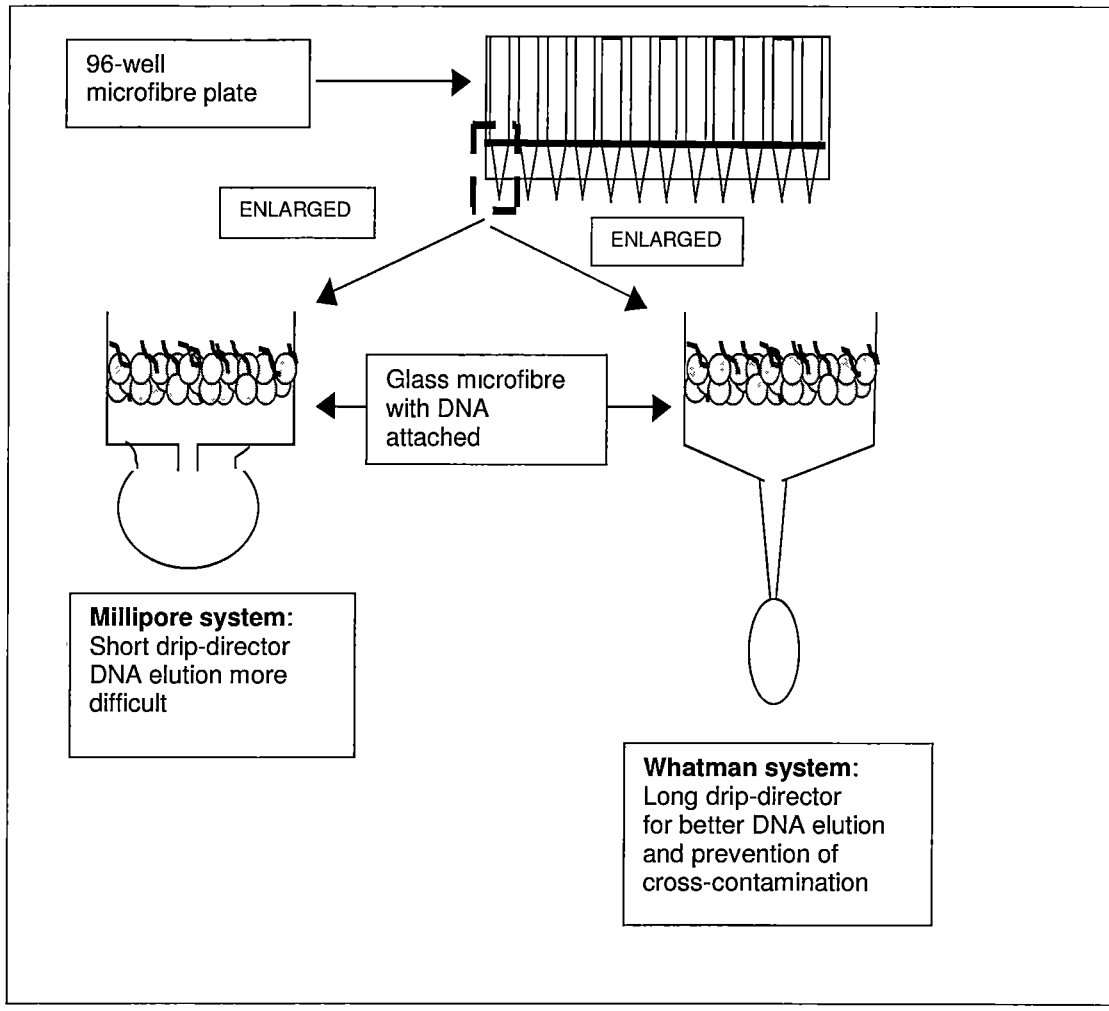


Figure 4.1: Difference in the elution conditions for Millipore and Whatman glass microfibre filter plates.

Quality of nucleic acids and the Whatman vacuum extraction system

Increasing the template volume from 1µl to 9 µl did not simply result in an increase in PCR sensitivity as was expected. A combination of factors were found to affect the ability to increase template volume without inhibiting PCR. The elution buffer recommended by Boom, *et al.* (1990), Tris-EDTA (10 mM Tris, 1 mM EDTA, pH 8), was found to cause inhibition when greater than 2.5 µl was used as

template for PCR. Larger template volumes were possible using 1 mM Tris buffer for elution but at volumes exceeding 6 µl PCR performance was compromised, see Figure 4.2. A dramatic five to 20 fold sensitivity increase was seen when 1.5 µg μl^{-1} BSA was added to the PCR reaction mix, also the intensity of the electrophoresis bands were greatly increased, see Figure 4.3. With the use of 1 mM Tris and 1.5 µg μl^{-1} BSA, a maximum volume of 6 µl of template DNA per 20 µl PCR reaction volume could be used. No increase in sensitivity was seen when BSA was added to the RT-PCR reaction mix, also adding BSA to the vacuum extraction protocol did not allow the addition of greater volumes of eluted RNA to the RT-PCR. However, with the vacuum extraction of RNA the washing of the nucleic acid prior to elution was very important. If the number of ethanol washes was reduced from 5 to 3, or the acetone step was omitted, 2 µl of RNA could be used in RT-PCR. However if 5 ethanol washes followed by 1 acetone wash were performed 5 µl of template RNA could be used in the RT-PCR assays.

During the seeding experiment designed to check for cross-contamination between the wells of the glass microfibre filter plate, cross-contamination occurred when a vacuum pressure of over 17 kPa was used during the first lysis step (using L6 lysis buffer). When a vacuum pressure of 13.5 kPa was used at this step no cross-contamination occurred.

Sensitivity of (RT-)PCR using the SEC- vacuum extracted nucleic acids

The sensitivity values for the PCR protocols were between 1 and 16 CFU per 200 µl of selective-enrichment medium, see Table 4.2 for details. Figure 4.4 shows the sensitivity pattern achieved for *L. garvieae* PCR; a similar pattern was seen for the other bacteria. Slightly more sensitive results were achieved using the RT-PCR system with sensitivity values of between 1 and 9 CFU per 200 µl of selective-enrichment medium achieved, see Table 4.2. Figure 4.5 shows the sensitivity pattern for *L. garvieae* RT-PCR; a similar pattern was seen for the other bacteria.

For *Y. ruckeri* and *L. garvieae* direct bacterial counts were also used to determine the sensitivity and to compare the results achieved by viable bacterial count. In this case the total number of bacterial cells in the inoculated selective-

enrichment media was 2-3 times greater than the viable counts for each bacterium, see Table 4.3.

When 1 x 10^9 CFU ml^{-1} of *Providencia* sp. was added to selective-enrichment broth that contained decimal dilutions of the target bacterium, sensitivity of the PCR and RT-PCR protocols, as determined be gel electrophoresis was not effected. This is demonstrated using *Y. ruckeri* PCR in Figure 4.6.

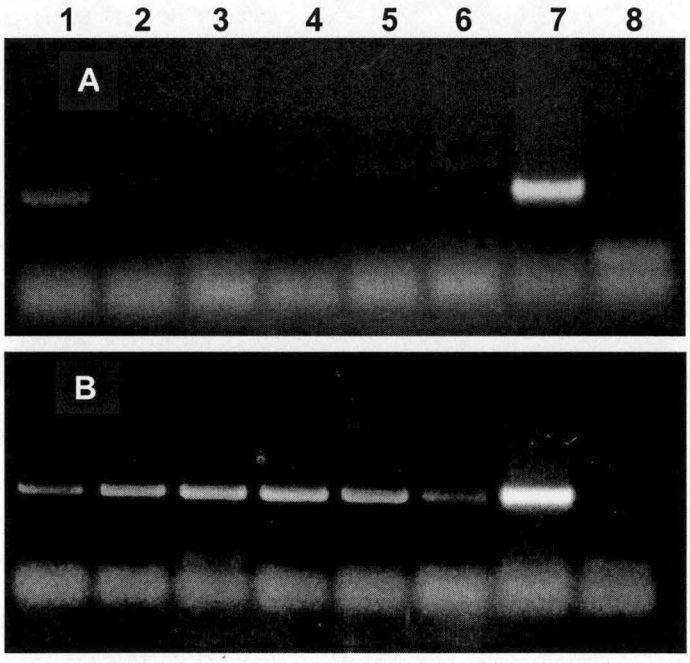


Figure 4.2: The inhibitory effects of different elution buffers (Tris-EDTA (TE) and 1 mM Tris, pH 8.0) and template volume on electrophoresis band intensity. Template: *Yersinia ruckeri* prepared in POST selective medium and DNA obtained by vacuum extraction, template concentration 150 CFU ml^{-1}. [A] TE buffer and [B] 1 mM Tris, pH 8.0. Lane 1, 1 µl template volume; lane 2, 3 µl template volume; lane 3, 4 µl template volume; lane 5, 6 µl template volume; lane 6, 7 µl template volume; lane 7, 10 ng μl^{-1} DNA positive control; lane 8, negative control.

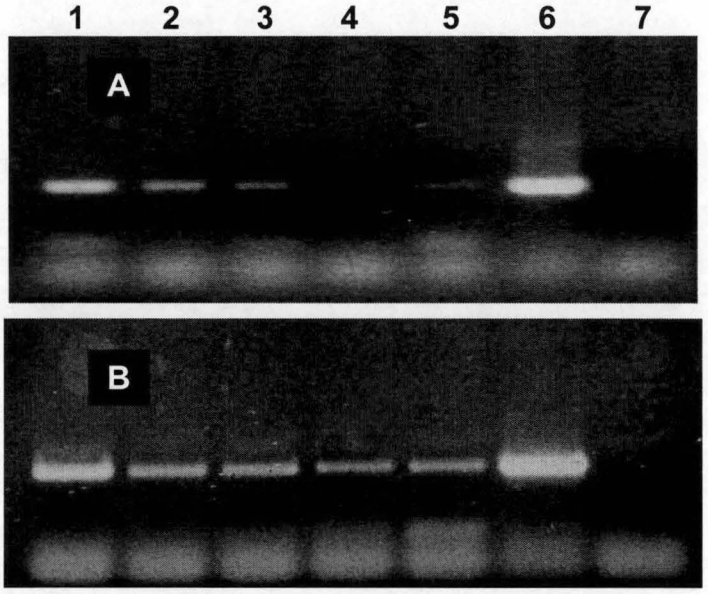


Figure 4.3: The effect of BSA on the ability to increase *Yersinia ruckeri* PCR template volume of vacuum extracted DNA. PCR with 1500 ng μl^{-1} BSA final concentration, [A] 1 µl template volume and [B] 9 µl template volume. Lane 1, 20 000 CFU $sample^{-1}$; lane 2, 2000 CFU $sample^{-1}$; lane 3, 200 CFU $sample^{-1}$; lane 4, 20 CFU $sample^{-1}$; lane 5, 2 CFU $sample^{-1}$; lane 6, 10 ng μl^{-1} positive control; lane 7, negative control.

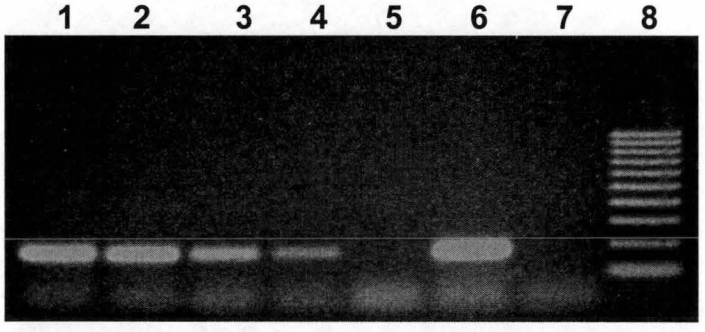


Figure 4.4: PCR sensitivity assay for *Lactococcus garvieae* extracted from SEC. PCR products (169 bp amplicon) of DNA from 10-fold dilutions of *L. garvieae* in selective-enrichment medium. Lane 1, 16 000 CFU per 200 µl sample volume; lane 2, 1600 CFU per 200 µl sample volume; lane 3, 160 CFU per 200 µl sample volume; lane 4, 16 CFU per 200 µl sample volume; lane 5, 2 CFU per 200 µl sample volume; lane 6, 10 ng μl^{-1} positive control; lane 7, negative control; and lane 8, Advanced Biotechnologies 100 bp molecular weight marker.

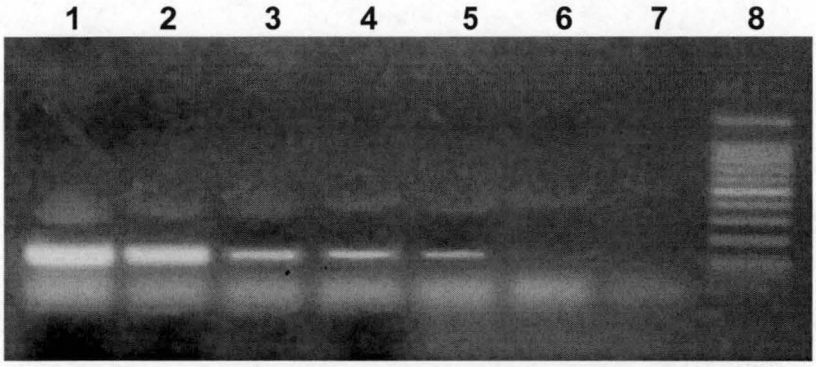


Figure 4.5: RT-PCR sensitivity assay for *Lactococcus garvieae* RNA extracted from SEC. PCR products (169 bp amplicon) from 10-fold dilutions of *L. garvieae* in selective-enrichment medium. Lane 1, 10 ng μl^{-1} RNA positive control; lane 2, 9000 CFU per 200 µl sample volume; lane 3, 900 CFU per 200 µl sample volume; lane 4, 90 CFU per 200 µl sample volume; lane 5, 9 CFU per 200 µl sample volume; lane 6, ≈1 CFU per 200 µl sample volume; lane 7, negative control; and lane 8, Advanced Biotechnologies 100 bp molecular weight marker.

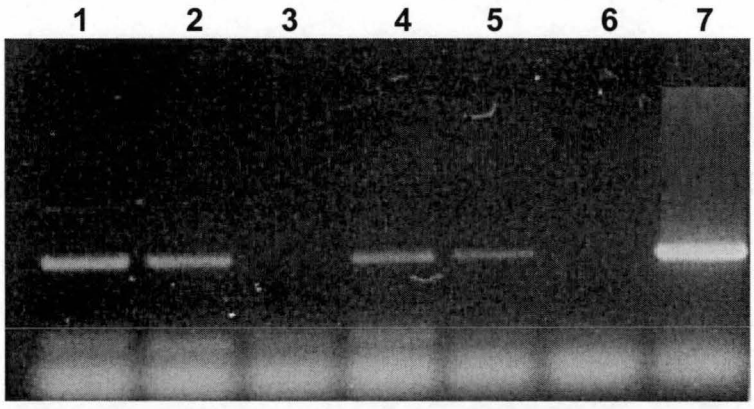


Figure 4.6: Effect of adding 1 x 10^9 CFU ml^{-1} of non-target bacteria (*Providencia* sp.) to decimal dilutions of *Yersinia ruckeri* on the sensitivity of the vacuum extraction-PCR protocol. Lane 1, 300 CFU ml^{-1} *Y. ruckeri*; lane 2, 30 CFU ml^{-1} *Y. ruckeri*, lane 3, 3 CFU ml^{-1} *Y. ruckeri*, lane 4, 300 CFU ml^{-1} *Y. ruckeri* and 1 x 10^9 CFU ml^{-1} non-target bacteria; lane 5, 30 CFU ml^{-1} *Y. ruckeri* and 1 x 10^9 CFU ml^{-1} non-target bacteria; lane 6, 3 CFU ml^{-1} *Y. ruckeri* and 1 x 10^9 CFU ml^{-1} non-target bacteria; lane 7, 10 ng μl^{-1} positive control.

Table 4.2 Detection limits of PCR and RT-PCR systems using nucleic acids extracted from SEC using the vacuum system.

Bacterium	Sensitivity of PCR as colony forming units (CFU) per 200 µl sample	Sensitivity of RT-PCR as colony forming units (CFU) per 200 µl sample
A. salmonicida	2	1
L. garvieae	16	9
T. maritimum	1	1
Y. ruckeri	1	1

Table 4.3: Detection limits of extraction PCR as determined by viable bacterial count (CFU) and direct bacterial count.

Bacterium	Viable bacteria in medium (CFU)	Total number of bacterial cells in medium
Y. ruckeri	1 CFU per 200 µl	3 cells per 200 µl
	5 CFU per ml	14 cells per ml
L. garvieae	16 CFU per 200 µl	34 cells per 200 µl
	80 CFU per ml	170 cells per ml

DISCUSSION:

Evaluation of two 96-well vacuum extraction units

The Millipore and Whatman 96-well systems both produced high-quality DNA from the selective-enrichment media. The decision to use the Whatman system was made because of the large sample volume capacity of the Whatman filterplates and the design of the 'drip-directors' underneath the filterplates which allow for easy nucleic acid elution. Whatman filterplates have a maximum total volume of 2 ml, therefore if one used 1 ml sample and 1 ml lysis buffer, nucleic acid would be collected from 1 ml of sample and concentrated into a final elution volume of about 30 µl. With the Millipore system a maximum sample volume of

100 µl could be used and, because of the design of the filterplates, a higher volume of DNA elution buffer (minimum of 50 µl) was required for efficient elution. A larger sample volume and a lower elution volume (due to the maximum volume of a PCR reaction) will theoretically produce a higher level of detection.

Efficacy of extraction protocol using Univac manifold and Whatman GF/B 96-well filter plates

Extraction of nucleic acids from SEC using GuSCN and glass mirofibre filter plates in a vacuum manifold proved to be very efficient. The system was well suited to the extraction of both DNA and RNA and could be optimised to extract remarkably small amounts of nucleic acid. The method is fast, about one hour for Gram negative bacteria, and less than 3 hours for Gram positive bacteria, and with a 96-well layout nucleic acids can be extracted from up to 96 samples simultaneously. The filter plates are available in 800 µl and 2 ml allowing for a large sample volume and, as elution is possible with very small volumes, the system concentrates the nucleic acids, increasing the likelihood of a positive result.

The efficient vacuum extraction protocol resulted after much optimisation. It is important that the lysis buffers are cooled to 4°C before use and that β-mercaptoethanol is added to the buffers just before use to avoid the breakdown of the RNA during extraction. The vacuum strength used during the removal of the L6 lysis buffer is of particular importance as this buffer contains triton X-100, which will cause foaming underneath the filter plate when too higher vacuum pressure is used. This foaming has the potential to cause cross-contamination between samples during extraction. When using less than half of the 96 wells in the filter plate during an extraction, the VacAssist membrane was essential for creating an even vacuum pressure across the filter plate. Uneven vacuum pressure sometimes resulted in inefficient flow of the buffers through the filter plate wells. Heating of the elution buffer was essential to remove all of the nucleic acids from the glass microfibre. After extraction, complete rehydration of nucleic acids overnight at 4°C was essential to avoid false-negative PCR results (Koller, *et al.* 2000), and was particularly necessary when the concentration of eluted nucleic acid was low. The

effect of inadequate rehydration was particularly evident with *L. garvieae* in the presence of co-extracted DNA from non-target bacteria.

Quality of eluted DNA

When added to the PCR reaction-mix at a ratio of 1:10, the eluted DNA obtained from the filter plate was suitable for PCR. While many DNA extraction protocols use Tris-EDTA (TE) as an elution buffer, high template volumes were not possible using this buffer as it inhibited the PCR. It is possible that the large amounts of EDTA added to the PCR mix with the Tris-EDTA were inhibiting amplification by chelating magnesium ions. Using 1 mM Tris to elute the DNA instead of TE rectified this problem. While a template volume of 6 µl was possible with DNA eluted with 1 mM Tris, PCR sensitivity was increased further by the addition of 1500 µg ml^{-1} BSA to the PCR reaction mix. Adding BSA at the concentrations used in other studies (400 - 670 µg ml^{-1}) (Romanowski, *et al.* 1993; Kreader 1996; Zhongtang and Mohn 1999) did not increase the sensitivity of the PCR or enable the addition of a greater template volume. BSA has been widely used to relieve PCR inhibition due to a wide range of substances (Kreader 1996). It is believed that BSA binds to particles such as lipids and anions (Loomis 1974) thereby preventing their binding and inactivation of *Taq* DNA polymerase. The improvement in PCR sensitivity with the addition of BSA to the reaction mix suggests the presence of PCR inhibitors in the nucleic acid elute. These inhibitors may have been due to carry over of salts during extraction (Boom, *et al.* 1999). Additional washing with ethanol during extraction did not alleviate this problem. In summary, if 1 mM Tris was used to elute the DNA and 1500 µg ml^{-1} BSA added to the PCR reaction mix it was possible to increase the ratio of eluted DNA in the PCR from 1:10 to 1:2.5 without evidence of PCR inhibition.

Quality of eluted RNA

The ability to add high volumes of RNA to the RT-PCR assays was affected by the Tris-EDTA buffer in the same way as the DNA PCR. However, adding BSA to the protocol did not noticeably increase the sensitivity of RT-PCR. In fact, titrations

that contained BSA in the reaction mix often had a lower sensitivity than those without BSA. When optimising the DNA extraction protocol more than 3 ethanol washes or an acetone wash step did not seem to decrease the presence of PCR inhibitors. In contrast, when extracting RNA, 5 ethanol washes and an acetone wash were essential in achieving high-quality RNA. Once optimised it was possible to add 5 µl of RNA to 15 µl of RT-PCR reaction mix.

Sensitivity of the protocol

The sensitivity values of 1-16 CFU per 200 µl sample volume for PCR and 1-9 CFU per 200 µl sample volume for RT-PCR achieved here compare favourably with similar protocols for extracting nucleic acids from enrichment media. Giesendorf *et al.* (1992) were able to detect 100 CFU *Campylobacter* spp. in 200 µl of selective medium, while Lindqvist (1999) could detect 1-2.0 x 10^4 CFU *Shigella* spp. in 200 µl of enrichment broth. PCR is capable of detecting non-culturable bacterial cells, therefore sensitivity values calculated with regard to viable cells alone (CFU), can be misleading. It can be seen from Table 4.3. that a sensitivity expressed in CFU appears better for the same experiment than a sensitivity giving the total number of cells present. With this in mind, the sensitivity of the extraction-PCR system was 3 cells for *Y. ruckeri* and 34 cells for *L. garvieae* per 200 µl of medium. The sensitivity achieved for *L. garvieae* was less than *Y. ruckeri* and was probably due to inefficient lysis. Increasing the concentration of lysozyme or lengthening the incubation time did not improve sensitivity.

In previous work the sensitivity of the PCR and RT-PCR using purified nucleic acids was found to be one bacterial cell (see Table 2.5), the values achieved with nucleic acids extracted from SEC using the vacuum system were not quite as sensitive. The main limitation to sensitivity in the vacuum extraction-PCR procedure is the inability to add all of the eluted nucleic acid to the one PCR reaction. During extraction, the DNA and RNA present in the 200 µl sample of enrichment-culture medium is concentrated into about 35 µl of nucleic acid elute, it is not possible to add this volume to one PCR reaction and it is not possible to decrease the volume for eluted nucleic acid.

When using a selective-enrichment medium with clinical samples, DNA from any non-target bacteria that can tolerate the selective components of that medium will be extracted at the same time as the target DNA. It has been reported that co-extracted nucleic acids can decrease PCR sensitivity (Giesendorf, *et al.* 1992; Swaminathan and Feng 1994; Weaver 1997). Using the extraction protocol described here, when 1 x 10^9 CFU ml^{-1} of *Providencia* sp. was added to selective-enrichment broth that contained the target bacterium, detection of the target bacterium was not compromised.

In conclusion, this method facilitates the high-throughput extraction of bacterial DNA and RNA from selective-enrichment culture media, in a form suitable for use in PCR and RT-PCR. The method enables the detection of very small amounts of a target bacterium, even in the presence of high levels of non-target bacteria. The use of vacuum filtration with a single 96-well glass microfibre filter plate allows for rapid and inexpensive (AU $0.55c per sample) extraction.

CHAPTER 5: Development of a Sensitive, High-Throughput One-Tube (RT-)PCR-Enzyme Hybridization Assay.

INTRODUCTION:

While optimised RT-PCR and PCR protocols are straightforward to perform, visualising the end product can impose a considerable burden where multiple samples are analysed. Small numbers of samples can be quickly and easily visualised by gel electrophoresis, but when large numbers of samples are being processed electrophoresis becomes cumbersome and time-consuming. Also, the diagnostic result produced from gel electrophoresis presumes that the PCR product has been produced specifically from the target nucleic acids. For applications such as disease diagnosis, an increased level of confidence can be achieved by hybridization with a specific internal probe to verify the amplicon sequence.

Probing a PCR product is usually performed using hybridization techniques such as dot or Southern blotting. While these methods work well, they are laborious and time-consuming. Micro-well ELISA-like hybridization methods which use the products of PCR (Nagata, *et al.* 1985; van der Vliet, *et al.* 1993; Gutierrez, *et al.* 1998) or RT-PCR (Barlic-Maganja and Grom 2001; Liolios, *et al.* 2001; Rey, *et al.* 2001) have been developed and form the basis of a convenient detection system. These methods can be significantly more sensitive than blotting and processing time is greatly decreased through shorter hybridization times and easier high-throughput sample processing. Such methods however require handling of PCR product when transferred into separate wells for hybridization, thereby increasing cost, time and importantly the risk of cross-contamination. A protocol for the amplification and verification of PCR amplicon in a one-tube system would be highly desirable, particularly for diagnostic applications.

Two types of PCR-Enzyme Hybridization tubes have been developed for one-tube amplification and detection of DNA. In 1991 Nunc developed a chemically derived surface called CovaLink™ (Rasmussen, *et al.* 1991). Sensitive one-tube PCR-Enzyme Hybdridzation assays (EHA) have been

developed using CovaLink™ tubes (Rasmussen, *et al.* 1991; Rasmussen, *et al.* 1994; Soumet *et al.* 1995), but problems with the thermostability of the resin were noted (Oroskar, *et al.* 1996). In 1996 an improved thermostable version of the CovaLink surface, NucleoLink™ (Nalge Nunc International, Naperville, IL, USA) was developed. NucleoLink tubes are made of an activated heat stable polymer that has a secondary amino group covalently grafted onto its surface. Prior to amplification, one of the PCR primers is covalently linked to the amino group by a carbodiimide mediated condensation reaction. Formatted in this way, a biphase PCR reaction can occur, with amplicon generated bound to the surface and free in liquid phase. The proprietary term for this process is the Detection of Immobilised Amplified Product in a One-Phase System (DIAPOPS) (Nalge Nunc International). While this one-tube EHA technology reduces the chance of cross-contamination by amplicon and potentially offers improved speed, specificity and sensitivity (Oroskar, *et al.* 1996), little has been published on its optimisation or practical application and to date there are no publications describing the use of NucleoLink to perform one-tube RT-PCR-EHA.

This work details the development and optimisation of PCR and RT-PCR EHA in NucleoLink tubes to produce a fast, sensitive, low cost, one-tube protocol to detect the presence of DNA or RNA from the four target pathogens. Internal probes are used to detect the amplified product and as a secondary specificity check. Visualisation is achieved using a biotin-streptavidin-alkaline phosphatase reaction and colour, generated from *p*-nitrophenylphosphate, is measured using an ELISA plate reader.

MATERIALS AND METHODS:

Positive controls

DNA extracted using the large-scale extraction technique and RNA extracted using an RNAAqueous™-4PCR (Ambion) extraction kit as described in Chapter 2 were used for positive controls.

Target nucleic acids from selective-enrichment media

Bacterial DNA and RNA from the selective-enrichment samples was extracted using the 96-well vacuum extraction method described in Chapter 4.

Determination of specificity

The organisms specified in Table 2.2 were used for specificity checks. Nucleic acids from these bacteria were extracted by suspending actively growing cultures in 100 µl of DEPC treated 18 Mohm water, boiling for 15 min, cooling rapidly to -20°C for 5 min followed by centrifugation for 10 min at 18000g. The nucleic acids (supernatant) were then transferred to a clean sterile tube.

DNase treatment of RNA

To achieve RNA free of DNA, the vacuum extracted nucleic acids were treated with DNase 1 enzyme (Promega) as detailed in Chapter 2.1.

Determination of sensitivity

Test sensitivity was determined at two levels, firstly with pure target DNA or RNA and secondly with target nucleic acids extracted from selective-enrichment media using the method detailed in Chapter 4. For the pure nucleic acids, serial dilutions were prepared from DNA or RNA of known concentration and these dilutions were amplified by (RT-)PCR. For the DNA or RNA from selective-enrichment media, a McFarland 0.5 suspension of bacteria in log-phase of growth was prepared in sterile water. From this a 1:1000 dilution followed by ten decimal dilutions using the selective-enrichment media as diluent, were prepared. Just prior to nucleic acid extraction, viable counts of the suspensions were determined by the Miles and Misra method (Miles, *et al.* 1938) using marine Shiehs medium (Appendix A) for *T. maritimum* and SBA for the other bacteria; plates were incubated for 48 hours at 25°C. Bacterial nucleic acids from each of the decimal dilutions were immediately extracted as described and the DNA or RNA amplified by (RT-)PCR-EHA.

16S rDNA primer modifications

For each PCR-EHA the 16S rDNA reverse primer was chosen as the solid-phase primer. Solid-phase primers were modified for use in NucleoLink tubes by an addition to the 5' end of a 10 base thymine linker and a terminal phosphate group as recommended by Nalge Nunc International.

Biotin labeled16S rDNA internal probes as described in Chapter 2 were used for hybridization to the solid-phase PCR products.

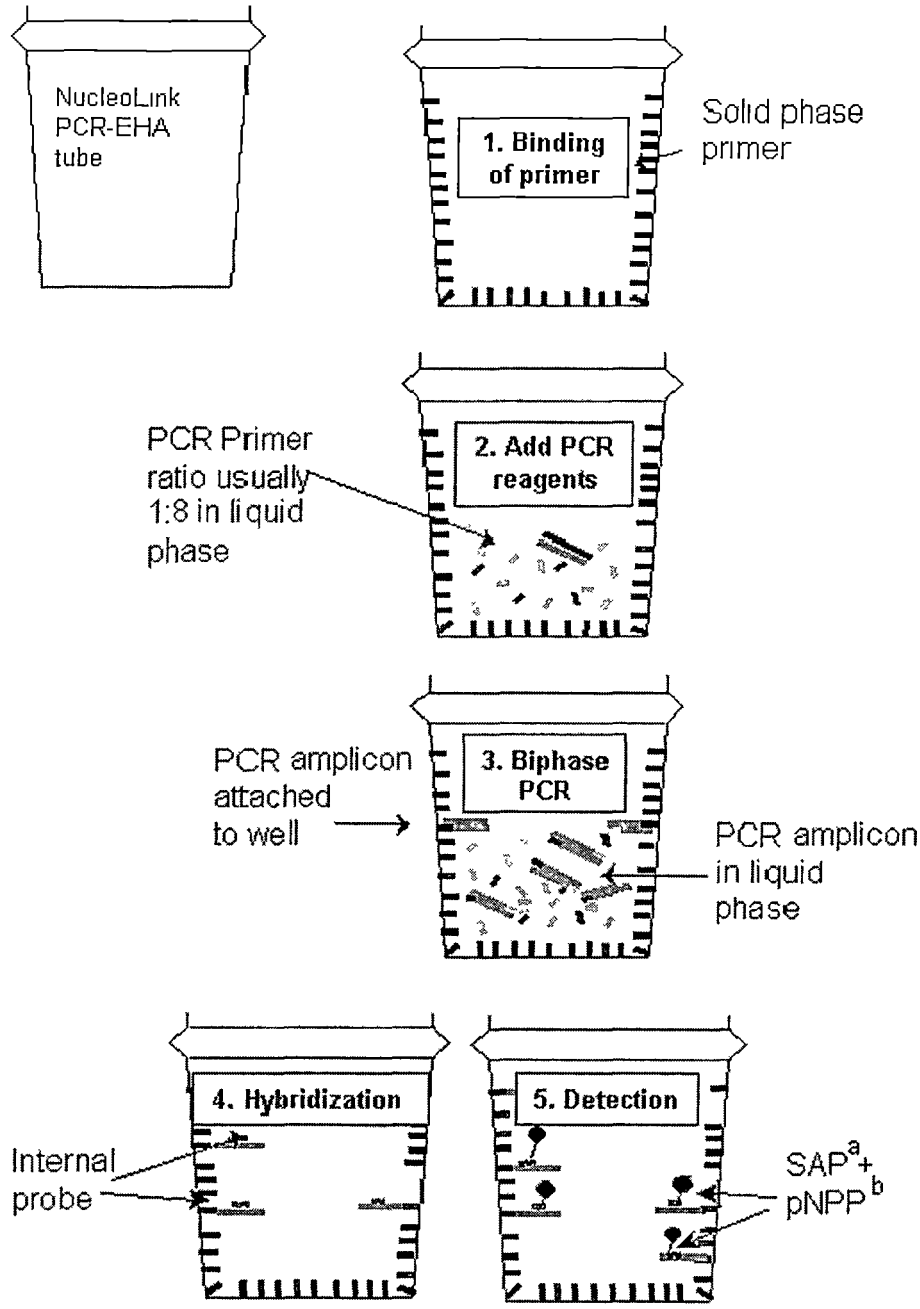
Biphase (RT-)PCR-EHA using the DIAPOPS technique

The DIAPOPS procedure involved five distinct steps. One, binding the solid-phase primer to the NucleoLink well wall. Two, adding the PCR reaction mix to each well. Three, biphase PCR. Four, hybridization with an internal probe and five, detection of solid-phase PCR with a conjugated enzyme and visualisation of the products colorimetrically. A diagrammatic representation of this procedure is given in Figure 5.1.

When performing biphase RT-PCR-EHA all solutions were made RNase-free by treating with 0.2% DEPC overnight followed by autoclaving.

Binding of solid-phase primer to micro-wells.

The modified reverse primers were covalently bound to the NucleoLink strips by the carbodiimide condensation reaction (CCR) as described for Covalink NH BreakApart^{TM} strips (Rasmussen, *et al.* 1994). CCR reagent sufficient for one strip was prepared by adding 1.63 mg of 1ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) to 840 µl of DEPC treated 18 Mohm water, 8.5 µl of 1 M 1-methylimidazole (1-MeIm) and 0.85 µl of 1000 ng μl^{-1} of solid-phase primer. NucleoLink strips were placed into a Nunc Frame (cat. no. 249182) and a 100 µl aliquot of CCR reagent solution was added to each well. The strips were sealed with Nunc Tape 8 (cat. no. 249719) and incubated at 50°C for 5 h. After incubation, non-covalently bound primer was removed by washing 3 times with 150 µl of pre-warmed 0.4 M NaOH with 0.25% Tween 20. The strips were further incubated at 50°C for 15 min followed by 3 more washes. The strips were then washed 3 times in DIAPOPS buffer (Appendix A), soaked for 5 min and then washed wells 3 more times. The wells were thoroughly emptied by



[a] Streptavidin alkaline phosphatase

[b] *p*-nitrophenylphosphate

Figure 5.1: Diagrammatic representation of biphase PCR in NucleoLink™ tubes.

tapping sharply several times while upside down on a paper towel. Strips could be stored in a clip seal plastic bag at 4°C for up to 2 months.

Asymmetric PCR technique.

NucleoLink wells were hydrated and blocked with 200 µl of DIAPOPS buffer with 10 mg ml^{-1} Fraction V BSA (Sigma-Aldrich) at room temperature for 1 h. The PCR reaction mix contained 200 µM each of dNTPs, 1.375 mM $MgCl_2$ for *A. salmonicida* and 2 mM $MgCl_2$ in all other cases, 1 X PCR buffer (Invitrogen), 10% glycerol, 0.1% Tween 20, 40 µg ml^{-1} IP10 (Cerus Corporation), 2 µM of the forward primer and 0.25 µM of the reverse primer, 0.5 units Platinum *Taq* DNA Polymerase (Invitrogen), 2 µl template DNA and sufficient 18 Mohm water to bring the total reaction volume to 20 µl. Optimum conditions for PCR cycling were: an initial 3 min denaturation at 94°C, followed by 35 cycles consisting of denaturation at 94°C (45 sec), annealing at 62°C (45 sec) for *L. garvieae* and *T. maritimum* ; annealing at 60°C (45 sec) for *Y. ruckeri* and *A. salmonicida*, and extension at 72°C (45 sec), with a final extension at 72°C for 3 min. A positive and negative control were included in each PCR run.

Reverse transcriptase technique.

NucleoLink wells were hydrated and blocked with 200 µl of DIAPOPS buffer with 10 mg ml^{-1} fraction V BSA (prepared in DEPC treated RO water <2 µS) and filter sterilized through a 0.2 µm filter) at room temperature for 1 h. The RT-PCR reaction mix contained 200 µM each of dNTPs, 1.375 mM $MgSO_4$ for *A. salmonicida* and 2 mM $MgSO_4$ in all other cases, 1 X RT-PCR buffer (Invitrogen), 0.1% DEPC treated Tween 20, 3 µM of each primer for *T. maritimum*, 2 µM each primer for *L. garvieae* and 2 µM forward primer and 0.25 µM reverse primer for *A. salmonicida* and *Y. ruckeri*, 0.2 µl Superscript™ One-Step RT-PCR with Platinum *Taq* (Invitrogen), 3 µl DNase treated RNA and sufficient 18 Mohm water to bring the total reaction volume to 20 µl. Optimum conditions for PCR cycling were: cDNA from RNA at 50°C for 30 min followed by a 3 min denaturation at 94°C, followed by 35 cycles consisting of denaturation at 94°C (45 sec), annealing at 62°C (45 sec) for *L. garvieae* and *T. maritimum*; annealing at 60°C (45 sec) for *Y. ruckeri* and *A. salmonicida*, extension at 72°C (45 sec), with a final extension at 72°C for 5 min. A positive

control, a no-sample and a no-RT enzyme negative control were included in each RT-PCR run.

PCR product inactivation.

For the PCR-EHA only, after amplification, the sealed strips were cooled to 4°C and then placed into a Nunc frame. The frame was placed directly onto a transilluminator (Ultra-Lum) and IP-10 activation occurred as described in Chapter 3.

Electrophoresis.

DIAPOPS amplification was assessed by gel electrophoresis of amplicon generated in the liquid-phase of the biphase PCR reaction. Electrophoresis was performed at 70 V for 40 minutes on a 2% (w/v) agarose gel prepared in TAE buffer and containing 0.5 µg ml^{-1} ethidium bromide (Sigma-Aldrich).

Denaturation of double-stranded amplicons.

Covalently bound amplicon was denatured to create single-stranded DNA for hybridization. Denaturing occurred by washing 3 times, soaking for 5 min and washing 3 more times in 0.2 M NaOH with 0.1% Tween 20 added just prior to use. After denaturation the wells were washed 3 times in DIAPOPS buffer, soaked for 5 min and then washed a further 3 times.

Hybridization with biotin labelled probes.

Denatured internal probe and salmon sperm DNA (Invitrogen) were diluted in hybridization buffer (Appendix A) to a final concentration of 50 nM and 100 µg ml^{-1} respectively, and 100 µl was added to each well. Hybridization was carried out at 50°C for 1 h. Unbound probe was removed by washing 3 times in 0.5 x SSC with 0.1% Tween 20, soaking for 15 min at 50°C and then washing 3 more times. After washing, the wells were thoroughly emptied by tapping sharply several times while upside down onto paper towel.

Colorimetric Detection of labelled probes.

Streptavidin alkaline phosphatase (Promega) was diluted 1:2000 in DIAPOPS buffer and 100 µl was added to each well. The strips were incubated

at 37°C for 1-2 h. After incubation the wells were washed 3 times, soaked for 5 min and washed 3 more times with DIAPOPS buffer. Then, 100 µl of 10 mg ml^{-1} p-nitrophenylphosphate (Sigma-Aldrich) diluted in 1 M diethanolamine containing 1 mM $MgCl_2$ (Appendix A) was added to each well. Colour development was allowed to proceed for 60 min in the dark at room temperature and OD readings were taken in an ELISA plate reader at 405 nm. If borderline positive-negative OD readings were obtained (values of about 1.2 times the negative control after 60 min) the reaction was allowed to proceed for up to 18 hours. After this time, samples with absorbance readings of at least 1.4 times the negative control corresponded to samples that contained template DNA or RNA. If required the EHA reaction was stopped by adding 100 µl of 1 M NaOH to each well.

RESULTS:

Sensitivity of the (RT-)PCR-EHA with pure nucleic acids

A sensitivity of 4 fg from pure DNA or RNA was achieved for PCR and RT-PCR for each bacterium. Typical results are shown with *Y. ruckeri* RT-PCR result shown in Figure 5.2. This level of sensitivity was only possible after extensive optimisation of the system for each bacterium. The most important factor in achieving optimum sensitivity were the primer ratios used in the PCR or RT-PCR. Optimum sensitivity was achieved for *A. salmonicida* PCR-EHA using 0.5 µM of the forward and 0.0625 µM of the reverse primer. However, comparable sensitivity was only achieved for *Y. ruckeri*, *T. maritimum* and *L. garvieae* when primer levels in the PCR were elevated to 2.0 µM for the forward and 0.25 µM for the reverse primer, four times the concentration used for *A. salmonicida* (see Figure 5.3).

Optimum results were achieved for *A. salmonicida* and *Y. ruckeri* RT-PCR-EHA using a primer ratio of 1:8 with 2 µM of the forward and 0.25 µM of the reverse primers, see Figure 5.4. This primer ratio resulted in inefficient RT-PCR-EHA when used with the other two bacteria. For *T. maritimum* and *L. garvieae* a 1:1 ratio of the two primers at a concentration of 3 µM each for *T. maritimum* and 2 µM each for *L. garvieae* gave the best results.

Establishment of EHA cut-off values

The ability to distinguish weak positive results from negative background readings was tested by running seven negative PCR reactions alongside a weak positive (4 fg of template DNA). In each case the weak positive gave a greater OD reading than the negative controls, with the positive reading usually 1.2 times greater than the mean value of the negative controls after 0.5 h as shown for *L. garvieae* in Figure 5.5. Occasionally weak positive results were not easily differentiated from the negative controls. In such cases extending the colour development period to 15 hours was sufficient to separate the weak positive reactions from the mean value of the negative controls by a factor of at least 1.4 (Figure 5.6).

Sensitivity of the SEC-(RT-)PCR-EHA

Once optimized for pure nucleic acids the (RT-)PCR-EHA was tested on DNA and RNA extracted from selective-enrichment media using the vacuum system described in Chapter 4. For PCR-EHA a sensitivity of 16 CFU per 200 µl sample volume was achieved for *L. garvieae*, 2 CFU for *A. salmonicida* and 1 CFU for *T. maritimum* and *Y. ruckeri*. The overall sensitivity of the RT system was slightly better than PCR-EHA, with detection limits between 3 and 9 CFU per 200 µl sample, see Table 5.1 for details. Figure 5.7 shows the sensitivity pattern for *T. maritimum*, a similar pattern was seen for the other bacteria.

Specificity of the (RT)-PCR-EHA

The high level of specificity achieved for the PCR and RT-PCR protocols (Chapter 2.1) was replicated using the NucleoLink PCR-EHA format. Clear, unambiguous reactions were evident, even with low concentrations of template DNA and RNA. For PCR-EHA there was total agreement between the presence of amplicon in the liquid-phase detected by gel electrophoresis and solid-phase amplicon detected by EHA (Figure 5.8). For RT-PCR-EHA positive EHA results were occasionally seen where gel electrophoresis bands were not evident. This only occurred when the sample was known to contain RNA, therefore in some cases the EHA was more sensitive than visualisation by gel electrophoresis.

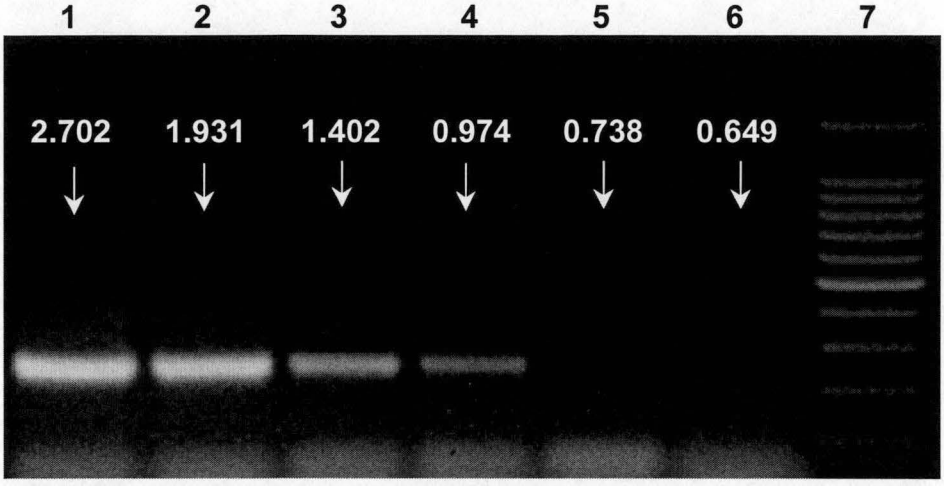


Figure 5.2: Sensitivity of biphase PCR with purified *Yersinia ruckeri* RNA. Assessment by agarose gel electrophoresis and corresponding EHA OD readings. Lane 1, 4 pg RNA; lane 2, 400 fg RNA; lane 3, 40 fg RNA; lane 4, 4 fg RNA; lane 5, 0.4 fg RNA; lane 6, negative control; lane 7, Advanced Biotechnologies 100 bp ladder.

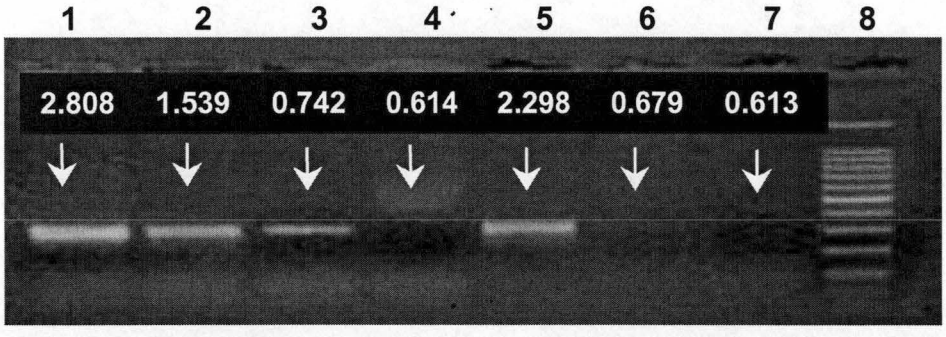


Figure 5.3: Sensitivity of biphase PCR with different concentrations of primer. Assessment by agarose gel electrophoresis and corresponding EHA OD readings. Lanes 1 to 3, 400 fg to 4 fg *Tenacibaculum maritimum* DNA, 2 µM forward primer, 0.25 µM reverse primer; lane 4, negative control; lanes 5 to 7, 400 fg to 4 fg *T. maritimum* DNA, 0.5 µM forward primer, 0.06 µM reverse primer; lane 8, Advanced Biotechnologies 100 bp ladder.

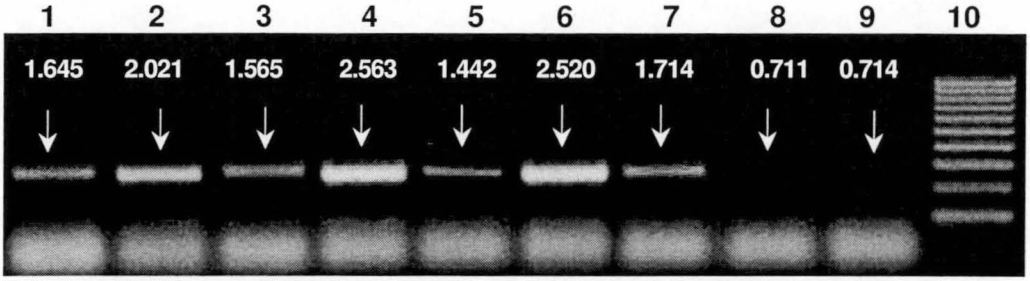


Figure 5.4: Effect of differing primer concentrations on agarose gel electrophoresis and EHA OD readings for *Aeromonas salmonicida* pure RNA. Lanes 1 to 7, 1 pg RNA: Lane 1, 2 µM each primer; lane 2, 2 µM forward primer, 1 µM reverse primer; lane 3, 1 µM each primer; lane 4, 1 µM forward primer, 0.5 µM reverse primer; lane 5, 0.5 µM each primer; lane 6, 2 µM forward primer, 0.25 µM reverse primer; lane 7, 2 µM forward primer, 0.5 µM reverse primer; lane 8, negative control with no RT enzyme; lane 9, negative control with no RNA; lane 10, Advanced Biotechnologies 100 bp ladder

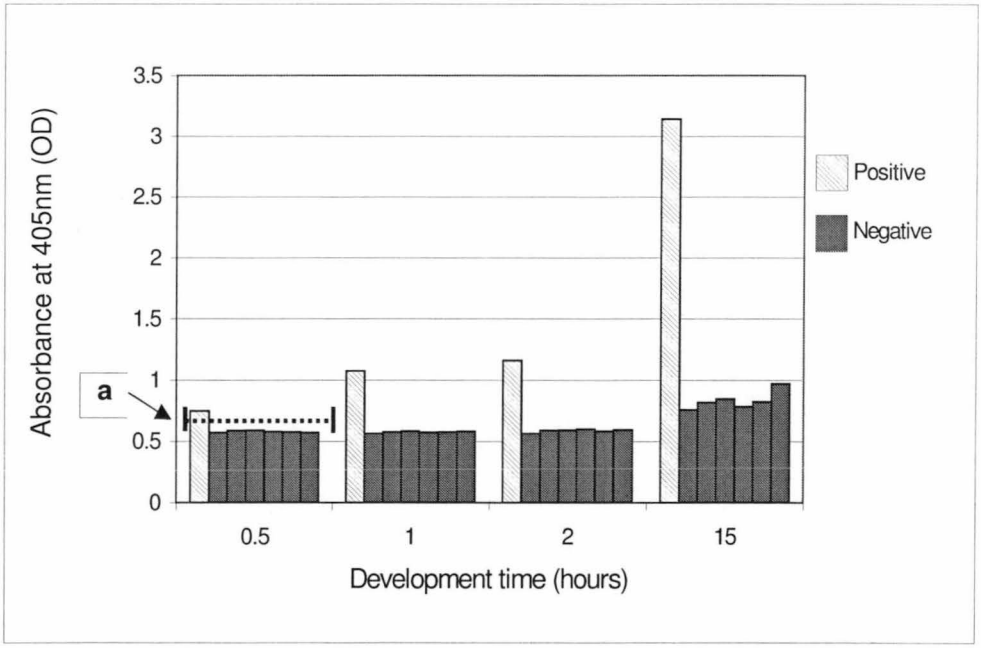


Figure 5.5: PCR-EHA absorbance values for *Lactococcus garvieae* showing the difference between weak positive OD readings (4 fg of template DNA) and seven negative OD readings over time. The weak positive reading (0.746) is more than 1.2 times the mean negative control value (1.2 x neg= 0.700) after 0.5 hours, shown by line (a).

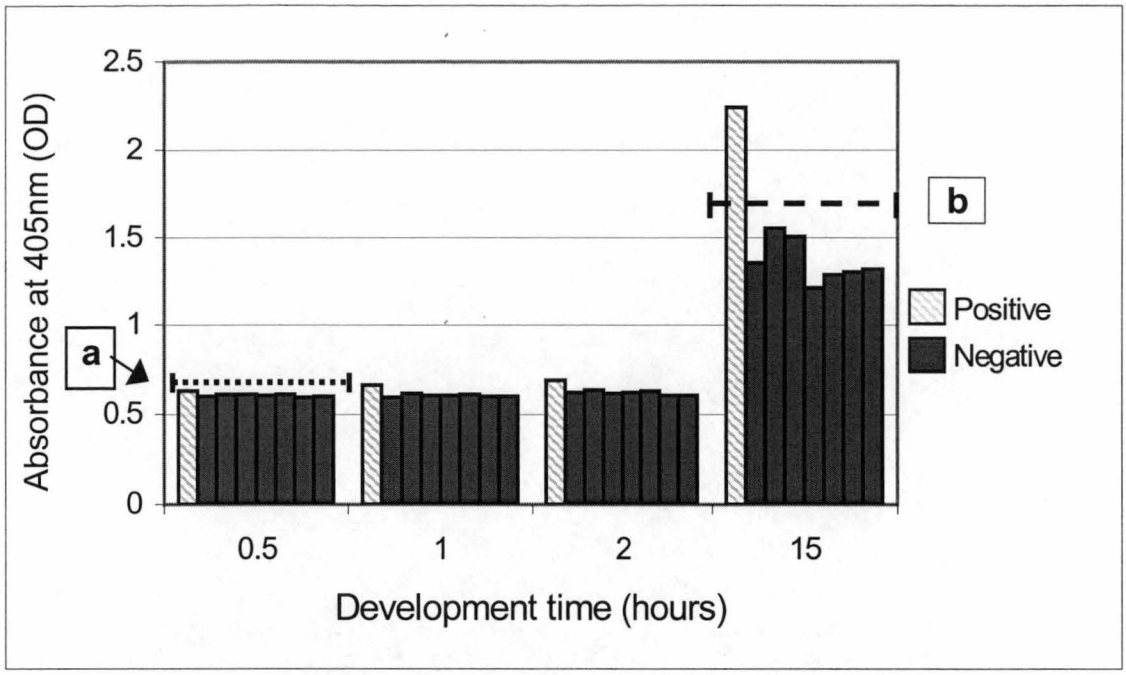


Figure 5.6: PCR-EHA absorbance values for *Aeromonas salmonicida* showing the difference between weak positive OD readings (4 fg of template DNA) and seven negative OD readings over time. In this case the weak positive reading (0.632) is less than 1.2 times the mean negative control values (1.2 x neg= 0.734) after 0.5 hours, shown by line (a). However, the weak positive (2.242) is distinguishable from the negative control by a factor of at least 1.4 (1.4 x neg= 1.697) after 15 hours incubation, shown by line (b).

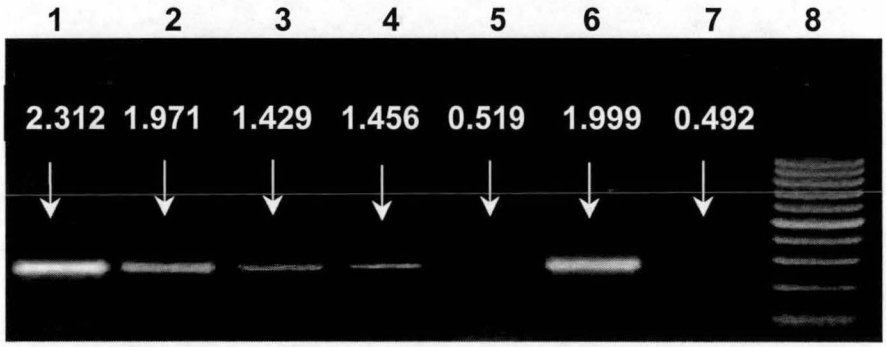


Figure 5.7: Sensitivity of biphase PCR with *Tenacibaculum maritimum* RNA extracted from enrichment culture medium. Assessment by agarose gel electrophoresis and corresponding EHA OD readings. Lane 1, 1000 CFU per 200 µl sample volume; lane 2, 100 CFU per 200 µl sample volume; lane 3, 10 CFU per 200 µl sample volume; lane 4, 1 CFU per 200 µl sample volume; lane 5, 0.1 CFU per 200 µl sample volume; lane 6, 10 pg positive control; lane 7, negative control; lane 8, Advanced Biotechnologies 100 bp ladder.

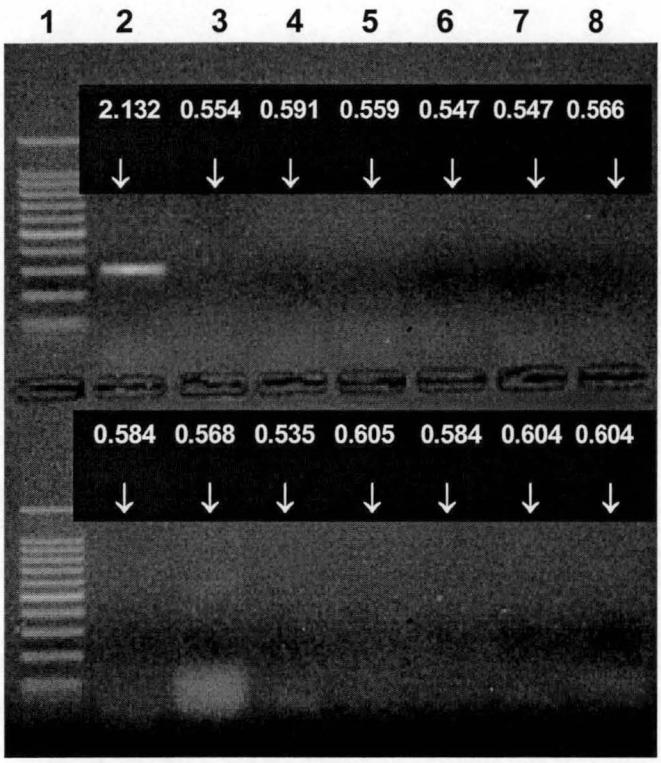


Figure 5.8: Liquid-phase PCR results and corresponding EHA OD readings showing the specificity of the *Tenacibaculum maritimum* PCR-EHA. Top row: Lane 1, Advanced Biotechnologies 100 bp ladder; lane 2, *Tenacibaculum maritimum* NCIMB 2154^{T}; lane 3, FCLB mucoid 96/5171; lane 4, FCLB mucoid 89/2244-9; lane 5, FCLB mucoid 89/2756-1; lane 6, FCLB mucoid CRC-2; lane 7, *Aeromonas salmonicida* 93/956-2; lane 8, *Flavobacterium columnare* NCIMB 2248^{T}. Bottom row: Lane1, Advanced Biotechnologies 100 bp ladder; lane 2, *Flavobacterium johnsoniae* ATCC 17061$^{Co\text{-}T}$; lane 3, *Vibrio anguillarum* 85/3475-1; lane 4, *Vibrio ordalii,* ATCC 33509^{T}; lane 5, *Vibrio splendidus* I, ATCC 25914^{T}; lane 6, *Cytophaga marinoflava* ACAM 75; lane 7, *Tenacibaculum ovolyticum* NCIMB 13127^{T}; lane 8, negative control.

Table 5.1: Comparative detection limits of SEC-(RT-)PCR-EHA.

Target bacterium	Detection limit of selective enrichment medium	Detection limit of DNA extraction as determined by PCR	Detection limit of SEC-PCR-EHA	Detection limit of RNA extraction as determined by RT-PCR	Detection limit of SEC-RT-PCR-EHA
A. salmonicida	3	2	5	1	3
L. garvieae	1[a]	16	16	9	9
T. maritimum	4[a]	1	4	1	4
Y. ruckeri	5[a]	1	5	1	5

All values are colony forming units per 200µl sample.

[a] Carson and Wilson 2001

DISCUSSION:

Sensitivity of the (RT-)PCR-EHA with pure nucleic acids

PCR performed in thin-walled PCR tubes (Scientific Specialties Inc., Ca, USA) achieved a sensitivity of 4 fg for each bacterium, see Table 2.5, Chapter 2. When biphase PCR was performed in the NucleoLink tubes using the same cycling conditions the sensitivity decreased to about 40 fg for each bacterium. This reduction in amplicon yield was attributed to the thicker walls of the NucleoLink tubes but could be completely restored to 4 fg when the denaturing and annealing times were increased from 30 sec to 45 sec per cycle. This sensitivity value compares favourably with the 100-300 fg of *Campylobacter jejuni* and *C. coli* DNA detected using NucleoLink by Grennan, *et al.* (2001).

Optimisation of the complete systems also required adjustments to the primer concentrations and ratio. For the complete (RT-)PCR-EHA system, optimisation was defined as the maximum sensitivity which gave agarose gel electrophoresis (liquid-phase PCR) and EHA readings that complemented each other. Reactions that gave a strong band and weak EHA readings or reactions that gave strong EHA and weak gel readings were considered sub-optimal. A conjugate ratio of 1:2000 was also critical in achieving maximum sensitivity. A lower conjugate ratio increased the background so that it was sometimes indistinguishable from weak positives and a higher ratio decreased the

sensitivity of detection. Optimisation of the development time resulted in two cut-off values, 1.2 times the mean value of the negative controls after 0.5 to 2 hours and 1.4 times the mean of the negative controls to a maximum of about 15 hours. Due to the occasional occurrence of weak positive readings not easily distinguishable from negative values after 0.5 hours every PCR-EHA assay was allowed to develop for 15 hours. This incubation period could not be extended beyond 18 hours, as after this time false-positive results could occur.

When performing PCR-EHA using nucleic acids extracted from selective-enrichment media the sensitivity achieved for *L. garvieae* was less than that achieved for the other bacteria. This was probably due to inefficient lysis during the extraction procedure, as discussed in Chapter 4. The PCR and RT-PCR sensitivity for the other 3 bacteria was also compromised compared with pure nucleic acids, most likely due to limitations of the DNA extraction procedure. The main limitation to sensitivity in the procedure is the ability to add only a proportion of the eluted nucleic acids to a single PCR reaction, as discussed in Chapter 4. Theoretically, if the total volume of eluted nucleic acids were able to be used in one PCR reaction, the sensitivity of the (RT-)PCR-EHA system for 200 µl of enrichment broth would be 4 CFU for *L. garvieae* PCR-EHA and 1 CFU for each of the other systems.

When the SEC-(RT-)PCR-EHA system is used with field samples, the final detection limit will be affected by any inhibitory effects of the selective medium on the target organism. For these selective media the inhibition was slight, and when added to the sensitivity limitation due to the DNA extraction technique forms the overall detection limit of the system, see Table 5.1.

Specificity of the PCR-ELISA protocol

The specificity of the PCR primers and corresponding internal probes have been established previously for *T. maritimum, L. garvieae* and *Y. ruckeri*. There was no reported evidence of cross-reaction with near related species, as defined by phenotype or genotype (Carson 1998). Also the same limitation of the *A. salmonicida* PCR was seen with the PCR-EHA system, i.e. cross-reaction with some strains of *A. hydrophila* and *A. bestiarum*. As a result of this limitation, positive *A. salmonicida* reactions should always be confirmed

by sequencing of the PCR amplicons or by performing biochemical profiling (Carson, *et al.* 2001) on the organism recovered in culture.

In conclusion, a highly sensitive and specific system that detects nucleic acids from the four target pathogens was developed. The system was optimised at every step to provide streamlined high-throughput sampling. The use of only one tube per sample from cDNA (for RT) to EHA decreases the cost and time involved in sample transfer and decreases the risk of cross-contamination between samples. The system is also rapid with the 96-well nucleic acid extraction to EHA results achieved in as little as 8 hours.

CHAPTER 6: Validation of the SEC-(RT-)PCR-EHA system with field samples

INTRODUCTION:

A PCR assay that has a high level of specificity and a low detection limit in the laboratory has a good chance of producing meaningful data when used on field samples. However, chemical and biological variables such as ions, skin mucus and faeces that can be present in field samples have the ability to inhibit PCR, decreasing test sensitivity. Also, when positive reactions occur, unknown and un-culturable organisms present in the sample (Pace 1997) can also place doubt in the test specificity.

It is recommended that validation of technology that uses PCR be conducted using predictive or comparative validation (Hiney and Smith 1998a). Predictive validation is used to study the relationship between a positive test result and the true disease status. Experimentally, the only recognised method for determining the true disease status of a population of fish is *in situ* amplification of the bacterium in the host, called the 'stress test' (Bullock and Stuckey 1975). The 'stress test' is not practical for validation of the SEC-(RT-) PCR-EHA as testing requires specialised facilities, is resource intensive and takes about three weeks to complete. Comparative validation requires that a new test be compared with two or more existing tests that have been previously validated. This form of validation also requires that the tests use totally different measurement principles, such as ELISA and direct culture, and is more effective when the tests have similar sensitivities.

Culture is the only method routinely used for the detection of the four target fish pathogens, and as SEC media have already been developed and validated (Carson and Wilson 2001) these media were used for comparison validation. Comparison validation was conducted by comparing positive SEC-(RT-)PCR-EHA reactions with the results obtained by sub-culture from the SEC broths. As there is only one test available for comparison, comparison validation formed only part of the field validation. Test specificity and sensitivity were also assessed from an epidemiological perspective. In epidemiology specificity is the proportion of animals without the pathogen that test negative (Cameron 2002).

To determine this, fish were sampled from farms with and without a history of disease (Smith 2001, C. Baldock AusVet Animal Health Services, Brisbane, pers. comm.). Test sensitivity from an epidemiological perspective is the proportion of animals which carry the pathogen and that test positive (Cameron 2002). As the proportion of animals that actually carry the pathogen is not known, test sensitivity was assessed by comparing the detection limit achieved in laboratory testing with the detection limit achieved by seeding field samples with low doses of the target organism and performing PCR-EHA.

MATERIALS AND METHODS:

PCR with *vap*A and PASS primer sets

PCR with *vap*A (Gustafson, *et al.* 1992) and PASS (Hiney, *et al.* 1992) primer sets were undertaken to follow-up positive *A. salmonicida* 16S rRNA gene SEC-PCR-EHA reactions.

PCR reactions were performed using parameters given by Byers, *et al.* (2002). Briefly, PCR reactions were performed in a 20 µl reaction containing 200 µM each of dNTPs, 2.5 mM $MgSO_4$ (1.375 mM for *A. salmonicida*), 1 X PCR buffer (Invitrogen), 0.5 units Platinum Taq DNA Polymerase (Invitrogen), 8 pM each of the two primers (see Table 6.1), filter plate extracted DNA, pre-warmed to 25°C and enough 18 Mohm water to bring the reaction volume up to 20 µl. PCR cycle conditions were 30 cycles of denaturation at 95°C for 30 sec., annealing at 57°C for 30 sec. And extension at 72°C for 90 sec. Positive and negative controls were included in each PCR run. After amplification amplicons were visualised by gel electrophoresis on a 2% (w/v) agarose gel containing 0.5 µg ml^{-1} ethidium bromide (Sigma-Aldrich) prepared in TAE buffer (Appendix A) and their size measured using a 100 bp molecular weight marker (Advanced Biotechnologies). The gels were run using a horizontal gel electrophoresis apparatus (Invitrogen) at 70 V for 40 minutes.

Table 6.1: *vap*A and PAAS PCR primer sequences for *A. salmonicida*.

PCR	Primer name	Primer	Primer sequence 5'→ 3'
*vap*A	AP 1	Forward PCR primer	GGC TGA TCT CTT CAT CCT CAC CC
	AP 2	Reverse PCR primer	CAG AGT GAA ATC TAC CAG CGG TGC
PAAS	PAAS 1	Forward PCR primer	CGT TGG ATA TGG CTC TTC CT
	PAAS 2	Reverse PCR primer	CTC AAA ACG GCT GCG TAC CA

Fish trials to test the dynamics of the high-throughput SEC-PCR system

The dynamics of the high-throughput system were evaluated and optimised by performing fish trials. Initial trials were conducted in May 2000 with Atlantic salmon from a freshwater farm with a history of *Y. ruckeri* infection. A volume of 2 L of POST selective-enrichment medium was prepared and dispensed into 10 ml volumes in sterile glass bottles. One 10 ml volume was inoculated with approximately 1 x 10^3 CFU of the target organism and incubated at 25°C overnight to check for growth in the medium. One hundred and fifty fish were lightly sedated and faecal samples extracted by gently squeezing the gut of the fish in the direction of the anus. The samples were placed into 10 ml of SEC medium. One hundred and twenty fish were returned to their cage and thirty fish were killed in order to extract a sample of spleen from the animal. Once collected the spleen samples were placed into 10 ml of SEC medium. All samples were then incubated at 25°C and DNA extracted after 5 days using the vacuum extraction technique detailed in Chapter 4. PCR was then performed on the extracted DNA (Chapter 4) and positive PCR reactions were checked for cell viability by sub-culture from the SEC medium. A second *Y. ruckeri* fish trial was conducted from the same farm two months later and 150 fish were sampled. To enable non-lethal testing, only faecal samples were taken from the fish. The samples were incubated at 25°C and DNA was extracted from the samples after 2 and 5 days incubation. To check the specificity of the SEC-PCR, 10 faecal samples from Atlantic salmon were collected from a farm with only low-grade disease outbreaks of *Y. ruckeri* prior to the time of sampling. After incubation at 25°C for 5 days in the SEC medium, vacuum extraction followed by PCR was performed on the samples.

Further testing of the dynamics of the system was undertaken with *L. garvieae* following a disease outbreak in NSW. This testing was performed after the rainbow trout had been treated and no longer showed signs of disease. A sample number of 133 fish were tested for *L. garvieae* carriage. For these fish 2.8 L of CORT selective enrichment medium was prepared and dispensed into 10 ml volumes in sterile glass bottles. One volume was inoculated with approximately 1 x 10^3 CFU of the target organism and incubated at 25°C overnight to check for growth in the SEC medium. Dissection was performed on the fish and about 200 mg of spleen or brain was placed in a 10 ml volume of SEC. The SEC samples were incubated at 25°C for 5 days. DNA was then extracted from the samples and PCR performed (Chapter 4). Positive PCR reactions were confirmed by sub-culture from the SEC medium onto SBA and incubating the agar plates for up to 5 days at 25°C.

Validation of the SEC-(RT-)PCR-EHA with field samples

Final validation of the SEC-PCR-EHA system was performed using samples from farmed salmonid fish. Where possible the fish trials were conducted from farm sites with recent history of disease, as it is impossible to effectively validate a system when no positive samples are present. The number of fish tested for each bacterium and the disease status of each farm is given in Table 6.2, and Figure 6.1 gives a diagrammatic explanation of SEC-PCR-EHA, and the procedure follows this example: For 100 fish, 750 ml of selective-enrichment medium was prepared and aseptically dispensed into 7 ml volumes in sterile plastic tubes. One 7 ml volume was inoculated with approximately 1 x 10^3 CFU of the target organism (see Chapter 2) and incubated at 25°C overnight to check for growth in the SEC medium. The medium was either used immediately or stored at –20°C until required, for a maximum of three months. When using the system to detect *T. maritimum*, *A. salmonicida* and *Y. ruckeri*, sampling was non-destructive. For *L. garvieae* testing, spleen or brain samples are required. To enable this fish were packaged on ice and sampled within 2 hours. After sampling, the SEC media were incubated at 25°C for 5 days. Vacuum extraction of nucleic acids (Chapter 4) and (RT-)PCR-EHA (Chapter 5) were then performed on the samples. In one trial for each of the target pathogens, 7 test-negative samples were seeded with

decimal dilutions of the target bacterium. This retrospective seeding was performed to ensure that the sensitivity of the system was not compromised due to any inhibitory substances in the field sample and to verify that the EHA cut-off values determined in the laboratory were applicable with field samples (1.2 times the negative control after 0.5-3 hours and 1.4 times the negative control after 4-15 hours development time). The number of bacteria added during the seeding was determined using the Miles and Misra method (Miles, *et al.* 1938) and vacuum extraction and PCR-EHA were performed on the samples.

As positive PCR reactions can occur from live and dead bacterial cells, during the trials any positive PCR reactions were tested for live bacterial carriage by RT-PCR-EHA (Chapter 5), and by sub-culture from the corresponding SEC medium. RT-PCR was used to approximate live bacterial carriage rather than PCR as RT-PCR detects RNA which is more labile than DNA, breaking down much sooner after cell death.

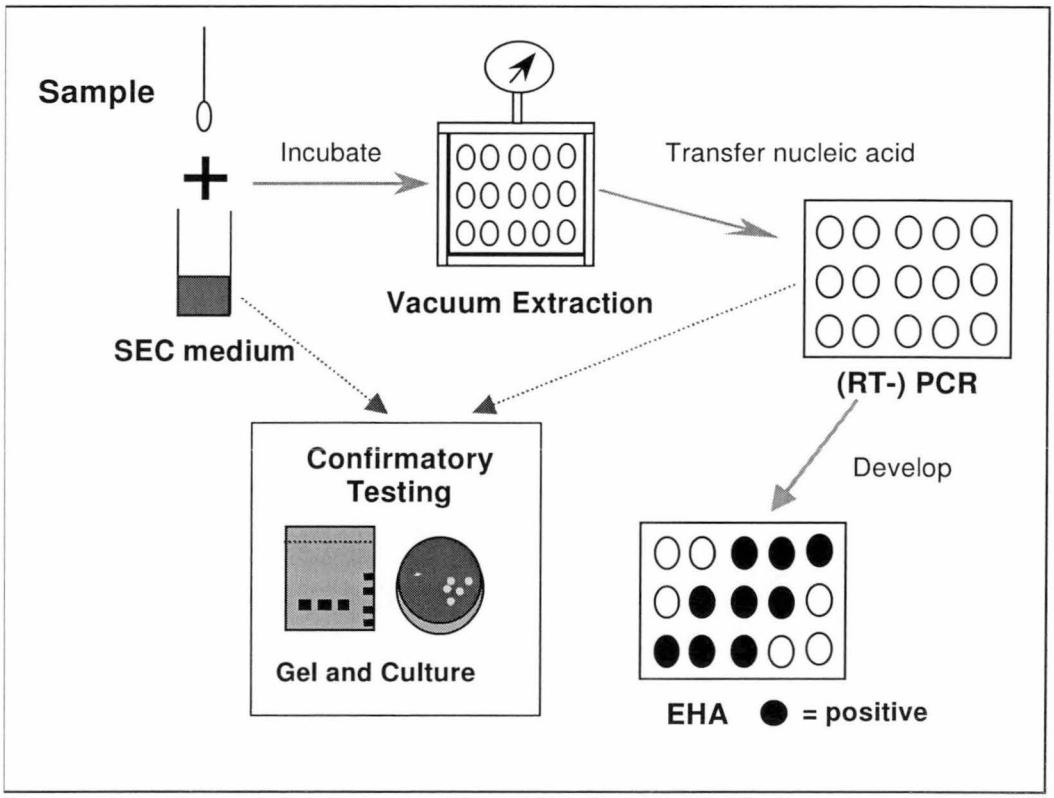


Figure 6.1: Schematic diagram of the SEC-(RT-)PCR-EHA.

The number of fish used to validate the system was determined using the epidemiology program Win Episcope 2.0 (de Blas, *et al.* 2000). A program that determines the minimum number of samples required to obtain a disease prevalence was used so that prevalence data could be obtained while validating the system. For the calculations, a population size of 1 million fish was assumed (however any population size over 5000 gives almost the same result), the accepted error was 10% and the level of confidence was 95%. Since prevalence data is unavailable for these pathogens and this information is likely to vary greatly depending on environmental factors, an expected prevalence of 50% was assumed for each fish population. Determining prevalence for a population with an expected prevalence of 50% requires the largest number of samples, (see Figure 6.2) and results in better precision (Baldock 2000). Using these parameters the minimum sample size used for the field trials was 96 (see Table 6.3). Where possible more than 96 fish were sampled as this allowed the accepted error (see Tables 6.3 and 6.4) to be lowered thereby achieving better precision.

In order to validate the specificity of the system, and especially to prove the validity of SEC-(RT-)PCR-EHA positive but culture-negative results, fish were sampled from non-infected areas with environmental factors close to those found in the infected areas. An expected prevalence of 10% was used for these trials with an increased level of confidence (97.5%) (see Table 6.4).

Table 6.2: Details of fish tested by SEC-PCR-EHA for each bacterium.

Bacterium	Fish trial #	Farm disease history	When tested	Number of fish tested by SEC-PCR-EHA	Sample source	Other details	Host fish	Total number of fish tested	RT system tested
A. salmonicida	1	No disease	February 2001	163	Skin mucus	Three age groups: 4 months post smolt 10 months post smolt Harvest fish	Atlantic salmon	478	No
	2	Disease 14 months previous	March 2001	130		18 months post smolt			
	3	Current disease	January 2002	184		See Table 6.5			
L. garvieae	1	No history of disease since 1991	August 2001	48	Spleen Brain	Average weight 900 g	Rainbow trout	181	No
	NA	Recent disease episode	May 2000	133*		Average weight 800 g			
T. maritimum	1	History of occasional disease	August 2001	96	Skin mucus	18 months post smolt	Atlantic salmon	242	Yes
	2		December 2001	96		9 months post smolt			
	3	No disease	January 2002	50		9 months post smolt			
Y. ruckeri	NA	History of disease	May 2000	150*	Faeces Spleen	Average weight 800 g	Atlantic salmon	400	Yes
	NA	History of low-grade disease	July 2000	10*	Faeces	Average weight 1200g			
	1	History of disease	June 2001	96		Average weight 800 g			
	2		January 2002	96		48 fish vaccinated 48 unvaccinated			
	3	No disease	July 2001	48		Average weight 900 g	Rainbow trout		

* SEC-PCR only

Table 6.3: Minimum number of samples required to determine disease prevalence in a population of fish, data from Win Episcope 2.0.

Population size	1000000
Expected prevalence (%)	50
Accepted error (%)	10
Level of Confidence (%)	95
Required sample size = 96	

Table 6.4: Minimum number of samples required to validate the specificity of the system, data from Win Episcope 2.0.

Population size	1000000
Expected prevalence (%)	10
Accepted error (%)	10
Level of Confidence (%)	97.5
Required sample size = 46	

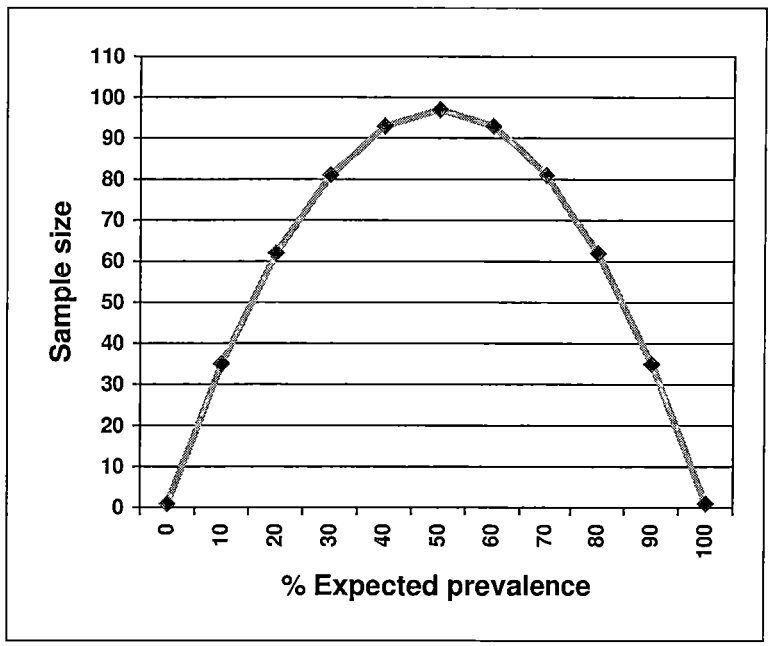


Figure 6.2. The relationship between expected prevalence and sample size; used when calculating the number of samples required to determine the true prevalence of a disease in a population.

RESULTS:

Fish trials to test the dynamics of the high-throughput SEC-PCR system

During the first *Y. ruckeri* fish trial, 14% of the 150 faecal samples and 10% of the 30 spleen samples were positive by SEC-PCR. Verification of the positive samples by sub-culture was only successful with 20% of the faecal samples. Those samples that were positive by SEC-PCR but not confirmed positive by sub-culture from the SEC had a significant amount of resistant normal flora that may have masked any colonies of *Y. ruckeri* that may have been present. The resistant normal flora were checked by PCR for cross-reaction to rule out the likelihood of false positives; no cross-reaction was found. The SEC-PCR positive results varied greatly in intensity as seen by gel electrophoresis (see Figure 6.3). Some bands were so weak that they were termed 'ghosts'. *Y. ruckeri* was not isolated by culture from any of the samples that produced 'ghost' bands. Verification of the positive spleen samples was successful, with *Y. ruckeri* isolated from all samples; there was no resistant normal flora isolated from the spleen samples. During the second *Y. ruckeri* fish trial 20% of the 150 faecal samples were positive by SEC-PCR after 5 days incubation. *Y. ruckeri* was not isolated by sub-culture from any of these positive samples. As seen in the first *Y. ruckeri* trial some of the SEC-PCR positive results showed very weak 'ghost' bands by electrophoresis. The SEC-PCR results also varied with incubation time. Samples that gave strong bands after 2 days incubation were also positive at 5 days, while some weak bands after 2 days incubation became stronger at 5 days. About 30% of the 'ghost' bands evident after 2 days incubation were not visible after 5 days incubation. Resistant normal flora were isolated by sub-culture from the SEC but no PCR cross-reaction occurred with these organisms.

Because of the number of un-confirmable positive SEC-PCR results, SEC-PCR was performed on 10 Atlantic salmon from a farm with a history of only low-grade *Y. ruckeri* infection. All of these samples were clearly SEC-PCR negative.

Figure 6.3: Variation in intensity of *Yersinia ruckeri* amplicon from faecal SEC samples, visualisation by gel electrophoresis. Row 1: lanes 1 to 8 are samples 1 to 8; sample 6 is clearly positive; in sample 5 the band intensity is so weak that it is described as a 'ghost'. Row 2: lanes 1 to 6 are samples 88 to 93; samples 88, 92 and 93 are positive; lane 7, 10pg μl^{-1} positive control; lane 8, negative control.

With the *L. garvieae* testing, a greater percentage of *L. garvieae* samples were positive by SEC-PCR than by culture; the same results as seen for the *Y. ruckeri* fish trials. Twelve of the 55 fish tested by PCR were positive, however only one of these fish was positive by sub-culture from the SEC medium. The 'ghost' phenomenon seen during the *Y. ruckeri* fish trials was apparent by gel electrophoresis, indicating weak PCR reactions. The disease prevalence was not determined, as SEC-PCR was not performed on every sample.

Validation of the SEC-(RT-)PCR-EHA with field samples

Each SEC-PCR-EHA, and where applicable the reverse transcriptase (RT) system, was validated using field samples. The *L. garvieae* system was validated using less samples than is ideal due to the lack of further disease outbreaks.

Aeromonas salmonicida.

SEC-PCR-EHA was performed on all 163 samples taken in the first *A. salmonicida* fish trial. No positive SEC-PCR-EHA reactions occurred from the sampled fish using the EHA negative cut-off value of 1.2 times the negative control proposed from laboratory experiments. Samples seeded with *A. salmonicida* gave positive SEC-PCR-EHA reactions down to 7 CFU ml^{-1} (the lowest dilution tested) indicating that there were no PCR inhibitors in the samples.

The second *A. salmonicida* trial produced very different results to the first. After allowing the EHA to incubate for 1 hour 12% (SE 2.23) of the samples had OD readings above 1.2 times the mean negative control cut-off value (0.781), which is the usual cut-off for the EHA (as determined in the laboratory trials). However using this value there was no clear difference between positive and negative EHA values with many of the readings having OD values very close to the cut-off, see Figure 6.4. To determine the appropriate cut-off value for data produced in a field trial, data such as EHA OD readings can be expressed on a continuous scale. When expressed in this way the results often form two distinct peaks, one due to the diseased individuals and one due to the healthy individuals (see Figure 6.5), the appropriate cut-off value will lie between these peaks. Often the results from the healthy and diseased individuals overlap, as in Figure 6.5. In this case the cut-off value requires a compromise between sensitivity and specificity to be made. The higher the cut-off value the greater the test specificity, however this compromises test sensitivity, resulting in a higher number of false-negative results. The opposite is true when the cut-off value is lowered. When the EHA data from this trial is expressed in this way there are two peaks in the data (Figure 6.6), however between these peaks the OD value is 0.68 which is only slightly above the negative control value (0.651) and significantly less than the typical cut-off value as determined in laboratory trials (1.2 times the negative control, 0.781). This value is unsuitable for use as a negative cut-off, therefore with this trial it is impossible to tell which samples are truly positive or negative after an EHA development time of 1 hour. When the EHA data was hard to interpret during the laboratory trials, further incubation resulted in values that were more easily interpreted. When the EHA assay was allowed to develop for a total of 15 hours the EHA readings were

more clear-cut. When, after 15 hours development time, these EHA values were expressed as continuous data (Figure 6.7), the EHA readings were easily interpreted with the data forming 2 clearly distinct curves with no cross-over. Between the curves was a clear gap, which corresponded to the usual cut-off value of 1.4 times the negative control (1.786). The clear cut-off value meant that no compromise was required between the test sensitivity and specificity, therefore there were no false-negative or false-positive results. Therefore according to SEC-PCR-EHA, *A. salmonicida* DNA was present in 30% (SE 7.88) of the Atlantic salmon tested. No *A. salmonicida* was recovered by sub-culture from the enrichment cultures although in all cases a resistant flora comprising mixed aeromonads was present. Because of the known cross-reaction of some *A. hydrophila* strains with the *A. salmonicida* 16S rRNA gene primer set, follow-up PCR was performed using the *vap*A and PAAS primer sets (Byers, *et al.* 2002). All tests with PAAS were negative but 19% of those tested were positive with the *vap*A primers. Screening of the resistant mixed aeromonads isolated from the enrichment cultures established that a strain of *A. hydrophila* was responsible for the positive *vap*A reactions. From this data it was established that there was no evidence that this group of fish were carriers of *A. salmonicida*.

A third *A. salmonicida* fish trial was conducted due to the coincidental occurrence of a disease outbreak in January 2002. In this trial SEC-PCR-EHA was performed on a total of 184 fish. Samples were collected from fish from 5 separate cages considered high risk, including some moribund or dead fish (cages 3 and 4). A smaller sample set was collected from apparently healthy fish from a nearby farm with no history of disease (see Table 6.5. for details). The fish sampled from the farm with no history of disease gave negative SEC-PCR-EHA results. By contrast, samples from dead or moribund fish were mostly positive. The remaining 155 fish were random samples which showed no clinical signs of disease, however significant levels of carriage were evident in all but one cage. Significantly the highest carriage level of 18% (SE 14.77) occurred in fish from cage 4 which also contained 9 of the dead or moribund samples. *A. salmonicida* was isolated by sub-culture from 5 of the 28 samples which has tested positive by SEC-PCR-EHA.

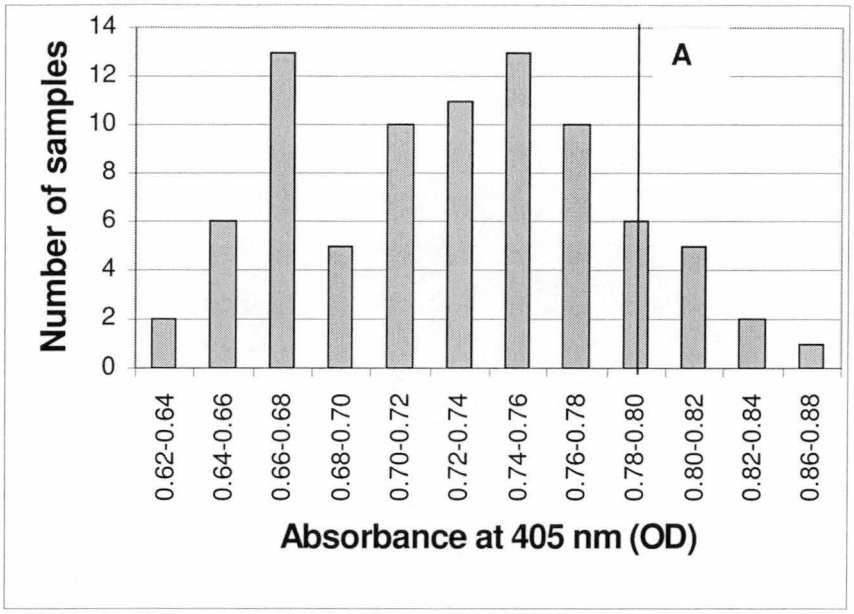


Figure 6.4: SEC-PCR-EHA absorbance values for *Aeromonas salmonicida* after 1 hour development time. Negative control is 0.651, positive control is 0.832. [A] represents the usual negative cut-off value after 1 hour development time (1.2 times the negative control, 0.781).

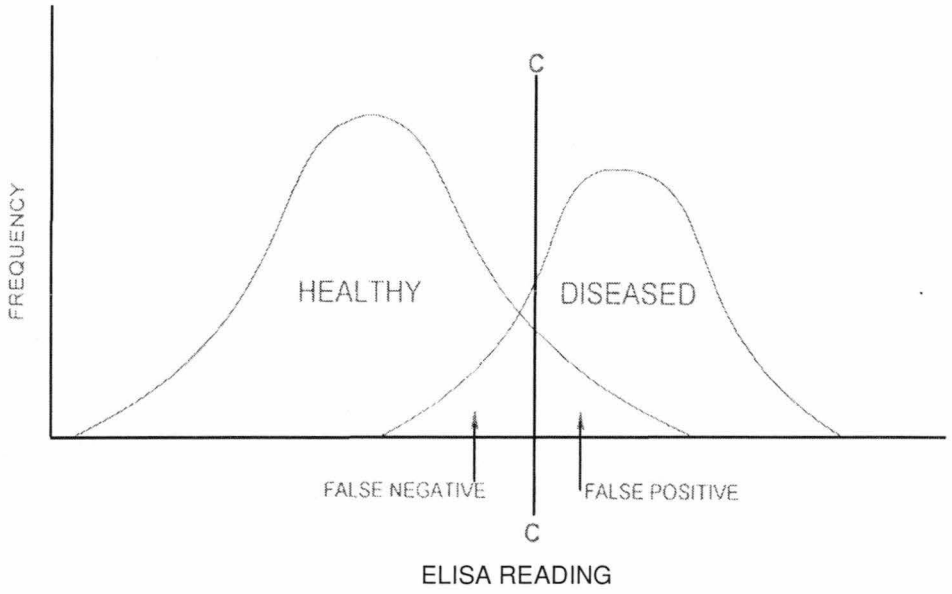


Figure 6.5: Frequency distribution of test results measured on a continuous scale for healthy and diseased groups of animals with a theoretical cut-off point separating reactors from non-reactors. (Cameron 2002).

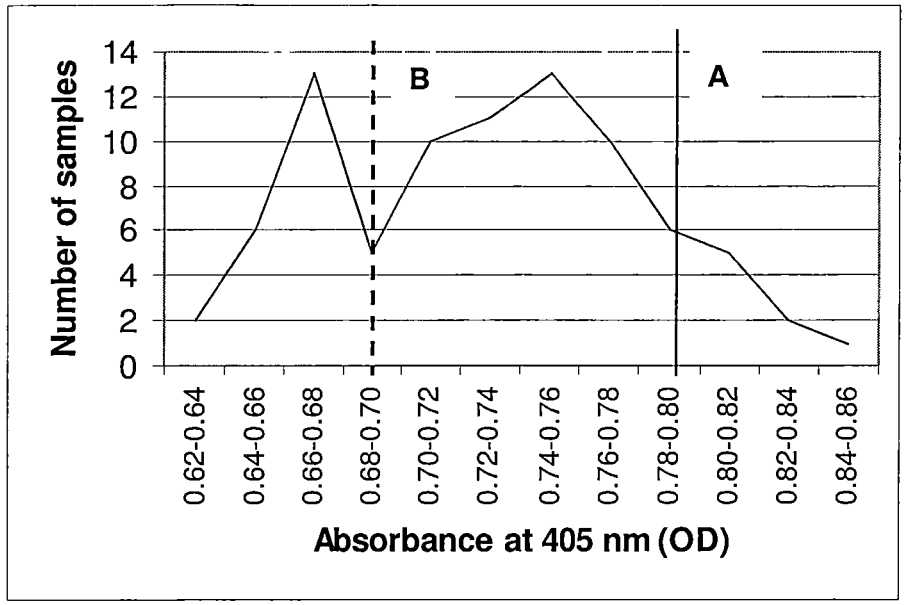


Figure 6.6: Frequency distribution of SEC-PCR-EHA absorbance values for *Aeromonas salmonicida* after 1 hour development time measured on a continuous scale. Negative control is 0.651, positive control is 0.832. [A] represents the usual cut-off value of 1.2 times the negative control (0.781), and [B] represents the value of the gap between the 2 peaks of data.

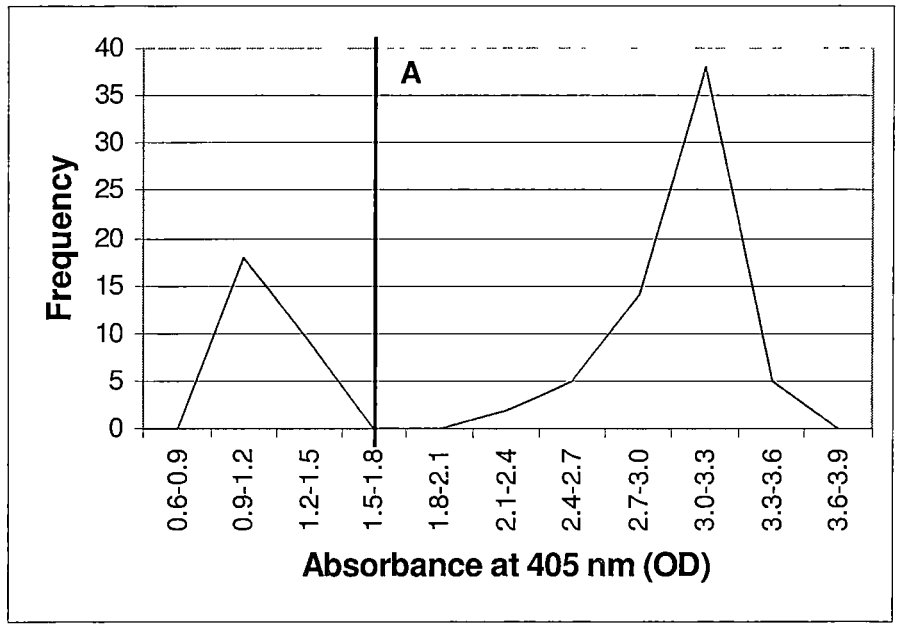


Figure 6.7: Frequency distribution of SEC-PCR-EHA absorbance values for *Aeromonas salmonicida* after 15 hours development time measured on a continuous scale. Negative control is 1.276, positive control is 3.429. [A] represents negative cut-off value of 1.4 times the negative control (1.786).

Table 6.5: Apparent prevalence of *Aeromonas salmonicida* during a disease outbreak, according to SEC-PCR-EHA; results by cage number and disease status.

Farm	Sample status	Number of samples	% SEC-PCR-EHA positive	Apparent prevalence
No history of disease	No clinical signs	3	0	0%
	Dead or moribund fish (cage 3 & 4, selected samples)	19	17	NA*
	Dead fish (cage unknown)	7	1	14%
History of disease	No clinical signs (cage 1)	30	1	3%
	No clinical signs (cage 2)	32	2	6%
	No clinical signs (cage 3)	30	1	3%
	No clinical signs (cage 4)	33	6	18%
	No clinical signs (cage 5)	30	0	0%

*Not applicable

Tenacibaculum maritimum.

During the first *T. maritimum* trial skin mucus samples were collected from near the pectoral fin and the SEC-PCR-EHA and reverse transcriptase (RT) systems used to determine disease prevalence. The SEC-PCR-EHA was used to determine a history of disease while the RT system was used to detect evidence of carriage of the live organism. SEC-PCR-EHA established a *T. maritimum* prevalence of 25% (SE 8.66), 16% were strong positives (EHA 1.4 times above the mean negative control) while the remaining 9% were between 1.2 and 1.4 times the negative control. No *T. maritimum* was isolated by sub-culture from the positive samples, however a large variety of *Flavobacterium*-like normal flora were recovered as resistant flora from the selective enrichment cultures. PCR on these resistant flora were all negative indicating that there were no cross-reactions and supports the belief that the

positive reactions detected by PCR-EHA were due to the presence of *T. maritimum* and not the resistant flora. Live *T. maritimum* carriage was tested using the RT-PCR-EHA system; only one sample was positive indicating 1% (SE 1.99) carriage of live *T. maritimum*. During this trial the RT-PCR-EHA system did not run as well as anticipated. The results of the EHA were only visible after over 6 hours incubation. The assay was repeated with the same result. Further testing determined the streptavidin alkaline phosphatase had decreased in potency with age, the problem was rectified by increasing the incubation time for this part of the assay from one to two hours.

A second *T. maritimum* trial was conducted using samples taken from the same farm as previously tested. Mucus samples were taken from 96 Atlantic salmon and the SEC-PCR-EHA and reverse transcriptase (RT) systems used to determine disease prevalence. Using SEC-PCR-EHA a carriage level of 7% (SE 5.1) was established. However no *T. maritimum* was isolated by sub-culture from the SEC medium. PCR was performed on any resistant flora but no cross-reactions were found. Follow up by SEC-RT-PCR-EHA of PCR positive samples showed the live carriage rate was only 1% (SE 1.99). Retrospective seeding was performed on 7 negative samples, positive reactions were detected down to 3 CFU ml^{-1} indicating that there were no PCR inhibitors in the samples.

To determine test specificity, especially since a number of the positive SEC-PCR-EHA results were not confirmed by culture, a small trial was conducted with fish from a sea farm with no history of *T. maritimum*. All SEC-PCR-EHA samples had absorbance values well below the 1.2 times the negative control cut-off value (see Figure 6.8). This trial gives confidence that the positive reactions from the previous trials were not due to non-specific cross-reactions.

Yersinia ruckeri.

The first *Y. ruckeri* fish trial used both the SEC-PCR-EHA and the reverse transcriptase (RT) systems to determine disease prevalence. By SEC-PCR-EHA a *Y. ruckeri* prevalence of 23% (SE 8.42) was determined using a negative cut-off value of 1.4 times the negative control after overnight development of the EHA. As seen in Figure 6.3. the strength of the band seen by gel electrophoresis varied greatly, with some bands weak enough to be called

'ghosts'. However during this trial no *Y. ruckeri* was isolated by sub-culture from the selective enrichment cultures. No cross-reaction was detected by PCR from resistant normal flora. Follow-up RT-PCR-EHA on the positive samples resulted in 4% (SE 3.92) live carriage of the bacterium. If RT-PCR and sub-culture are considered reliable indicators of live carriage, the prevalence found using each method should be similar. In cases such as this, where there is a discrepancy between the values, and it is unknown which test is correct, true prevalence is hard or even impossible to determine. Performance of the RT-PCR-EHA compared with sub-culture from the enrichment broths is clearly displayed in a 2 x 2 table (Cameron 2002), see Table 6.6. The observed proportion of agreement (de Blas, *et al.* 2000) between the two tests was 96% with 95% confidence. Disagreement between the two tests was always due to RT-PCR positive and culture negative results.

Table 6.6: *Y. ruckeri* RT-PCR-EHA and sub-culture from SEC comparison matrix

	Culture		
RT- PCR-EHA	+	-	Total
+	0	4	4
-	0	92	92
Total	0	96	96

The second *Y. ruckeri* trial was conducted in January 2002 from the same farm as originally tested. Faecal samples were taken from 48 fish that had been vaccinated against *Y. ruckeri* and from 48 fish of the same age that had not been vaccinated. The *Y. ruckeri* SEC-PCR-EHA and reverse transcriptase (RT) systems were used to determine history of infection and evidence of active carriage of the bacterium. By SEC-PCR-EHA 4% (SE 5.54) of the vaccinated fish showed a history of infection, no *Y. ruckeri* was detected by follow-up SEC-RT-PCR-EHA suggesting that the fish no longer actively carried the pathogen. By SEC-PCR-EHA 8% (SE 7.67) of the unvaccinated fish showed a history of infection and 4% (SE 5.54) were determined to be active carriers. Sub-cultures were taken from enrichment media that was positive by PCR and RT-PCR; all cultures were negative for *Y. ruckeri*.

A large number of *Y. ruckeri* positive SEC-PCR-EHA results could not be confirmed by culture, a characteristic also found when testing sea-farmed Atlantic salmon for *T. maritimum*. In order to test for non-specific cross-reactions, a small-scale trial was conducted with rainbow trout from a farm that co-cultures Atlantic salmon. While there had been frequent episodes of yersiniosis in the Atlantic salmon there had not been any outbreaks in the rainbow trout. Rainbow trout were used because of their inherent resistance to infection with *Y. ruckeri* serotype O1b. Figure 6.9. shows the distribution of the absorbance values resulting from the SEC-PCR-EHA. It can be seen that all but one of the EHA absorbance readings were well below the negative cut-off of 1.4 times the negative control OD value after 15 hours incubation. Sub-culture from the SEC broth that gave the positive EHA result resulted in the isolation of *Y. ruckeri* from the sample. Decimal dilutions of a suspension of *Y. ruckeri* were added to the selective-enrichment cultures to check for false-negative reactions due to PCR inhibitors. A positive SEC-PCR-EHA result was achieved with retrospective seeding at less than 1 CFU per 200 µl sample, showing the high sensitivity of the system and indicating that there were no PCR inhibitors present.

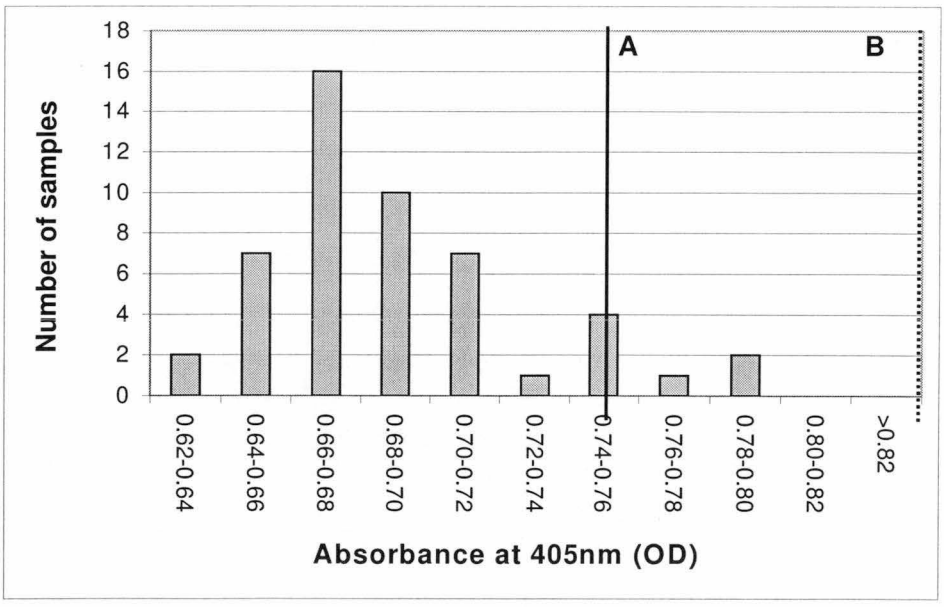


Figure 6.8: *T. maritimum* SEC-PCR-EHA absorbance values for 50 skin mucus samples from a farm with no history of disease. [A] represents the value of the negative control after 0.5 hours development time (OD = 0.742), [B] is the negative cut-off value at 1.2 times the negative control (OD = 0.871).

Lactococcus garvieae.

The last recorded outbreak of disease due to *L. garvieae* in Tasmania occurred in 1991. In the absence of disease a large-scale fish trial was not possible. However validation of test specificity with field samples was achieved by testing spleen samples from 48 rainbow trout by SEC-PCR-EHA. The SEC-PCR-EHA results were all negative after 3 hours development time using 1.2 times the negative control OD value as the negative cut-off, and after 15 hours using 1.4 times the negative control as the cut-off. Decimal dilutions of *L. garvieae* were added to the selective-media to check for false-negative reactions due to PCR inhibitors. The level of detection by SEC-PCR-EHA with retrospective seeding was 8 CFU 200 μl^{-1} sample, showing the high sensitivity of the system and indicating that no PCR inhibitors were present in the DNA elute.

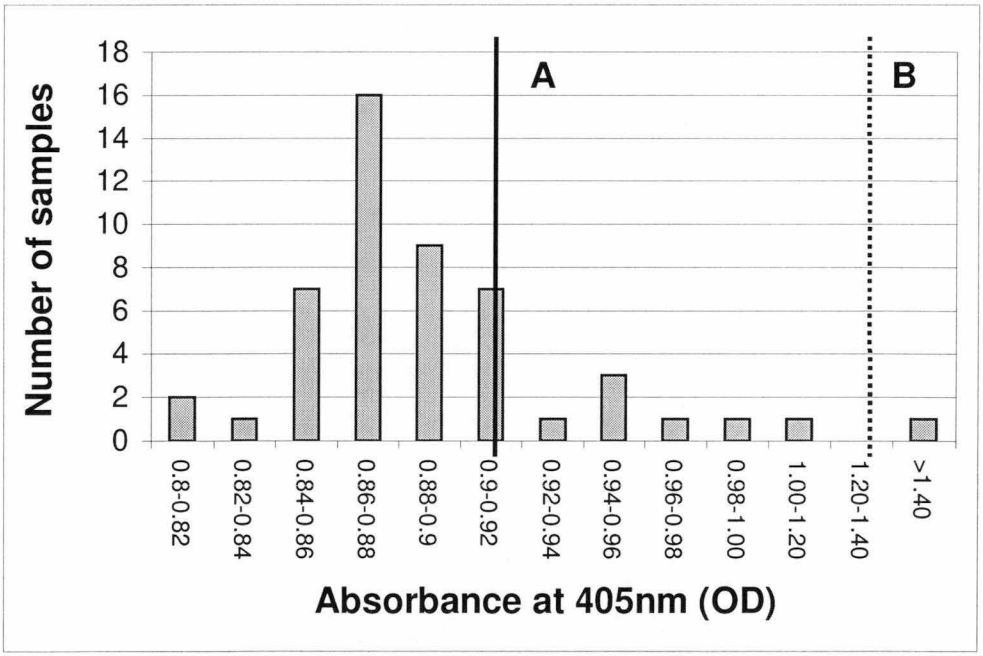


Figure 6.9: *Y. ruckeri* SEC-PCR-EHA absorbance values for 48 faecal samples from a farm with no history of disease. [A] represents the value of the negative control after 15 hours development time (OD = 0.901), [B] is the negative cut-off value at 1.4 times the negative control (OD = 1.26).

DISCUSSION:

Fish trials to test the dynamics of the high-throughput SEC-PCR system

The SEC-PCR system worked well during the field trials, with the high-throughput vacuum extraction and PCR protocols proving efficient for processing large numbers of samples. During these initial trials many positive PCR reactions could not be verified by sub-culture, either due to resistant flora overgrowth, or no growth. Due to this result it was evident that PCR and sub-culture from the SEC media are quite different in test sensitivity and/ or specificity, thus comparison validation was not possible between the two tests. Due to the high sensitivity of PCR it is possible that some of the positive PCR reactions were false-positive results. Possible causes of positive PCR reactions are: cross-contamination from other positive samples; contamination due to amplicon from previous amplifications (Fahle, *et al.* 1999; Vaneechoutte and Van Eldere 1997); amplification of DNA from un-culturable cells or fragments of the target pathogen (Miriam, *et al.* 1997; Waage, *et al.* 1999) or amplification of DNA from un-culturable non-target organisms that are present in the sample (Pace 1997). During the field trials positive results were not due to cross-contamination from other positive samples. During the development of the vacuum extraction protocol (see Chapter 4) positive and negative samples were extracted side-by-side and when the extraction protocol was strictly followed no cross-reaction occurred. Positive reactions were not due to amplicon carry-over as strict containment policies were followed at all times (Kwok and Higuchi 1989), the PCR protocol incorporates the amplicon inactivating chemical IP-10 (see Chapter 3) and positive reactions were not seen in the negative controls. Therefore, it is most likely that the positive PCR reactions were due to amplification of DNA from un-culturable cells or fragments of the target pathogen. However, when positive PCR reactions can not be confirmed by culture whether the target bacterium was the cause of positive PCR reactions can not be categorically proven. In this case one has to resort to proving that the positive PCR reactions are true beyond reasonable doubt. Some of the practical observations support the hypothesis that positive PCR reactions are due to the presence of the target pathogen. When the culture-negative, PCR-positive reactions were visualized by gel electrophoresis the band strength was

almost always much fainter than the bands produced from positive reactions that could be confirmed by culture. PCR results obtained from DNA extracted after the selective media had been incubated for 2 and then a further 3 days showed a 30% reduction in the number of 'ghost' bands, possibly the result of degradation of the DNA fragments in the selective-media over time. In contrast, clearly evident bands at 2 days remained so after 5 days incubation, and the samples could be confirmed by sub-culture. To increase the confidence in the specificity of the system, Atlantic salmon samples were collected from a farm with only low-grade disease outbreaks of *Y. ruckeri* preceding the time of sampling. Ideally the specificity should be tested on Atlantic salmon from a non-infected site, unfortunately however this does not exist. As the samples were taken from a farm with similar water quality and temperature to the farm that has a long history of disease due to *Y. ruckeri*, it was presumed that the normal fish flora would be similar and that any false-positive PCR reactions due to un-culturable non-target organisms would repeat themselves. No PCR or culture positive reactions occurred. Given the evidence, it is therefore concluded that positive PCR reactions are likely to be the result of detecting the target pathogen.

Validation of the SEC-PCR-EHA and RT systems with field samples

Aeromonas salmonicida.

Validation of the *A. salmonicida* SEC-PCR-EHA system in the field was undertaken using 315 fish from farms with some history of disease and 163 fish from farms with no history of disease. To determine field sensitivity retrospective seeding was used and the sensitivity compared with that achieved using purified DNA in the laboratory. A sensitivity of 1 CFU per 200 µl sample was achieved indicating that there were no PCR inhibitors in the nucleic acid sample. Test specificity also correlated with laboratory testing. Negative EHA results were achieved from the Atlantic salmon that had no history of disease, therefore all fish without the pathogen tested negative. Therefore from an epidemiological perspective the results of this test suggest that non-infected fish will be correctly identified. However from laboratory testing the *A. salmonicda*

16S rRNA PCR is known to cross-react with some strains of *A. hydrophila* (Carson 1998). This specificity problem was highlighted in the second fish trial where the apparent 30% prevalence of *A. salmonicida* was due entirely to the presence of *A. hydrophila*. The presence of *A. hydrophila* was confirmed by sub-culture from the SEC. It was expected that PCR using the *vap*A and PAAS primer sets would be negative as these assays are reported to have a combined specificity of >99% (Byers, *et al.* 2002). All of the samples were negative by PAAS PCR, however 19% of the samples were positive by *vap*A PCR. It was established that two strains of *A. hydrophila* were present in the enrichment cultures. Both strains of *A. hydrophila* cross-reacted with the 16S rRNA primers and the second strain cross-reacted with the *vap*A primer set as well. The amplicon was sequenced to confirm that it had been derived from the *vap*A gene, and was not the result of a non-specific reaction. Sequencing confirmed that the amplicon had a sequence expected for the *vap*A gene, and that this strain of *A. hydrophila* has the complete *vap*A gene (N. Gudkovs, Australian Fish Disease Laboratory, CSIRO). It is apparent that the *vap*A gene can no longer be considered specific for *A. salmonicida*, an important implication for the identification and detection of the pathogen. The occurrence of this cross-reaction may place doubt in the usefulness of the 16S rRNA primer set for detecting *A. salmonicida*, but due to the ability of these primers to detect every possible strain of *A. salmonicida* their use is justified for population screening where there is no evidence of disease. Some other primer sets are more specific but these do not detect all strains of *A. salmonicida*. As a consequence of this trial, the *A. salmonicida* selective-enrichment medium was re-formulated to inhibit the growth of these *A. hydrophila* strains, the medium was called HK3C, the development of the medium is detailed in Chapter 2.3.

The third *A. salmonicida* fish trial used the SEC medium HK3C, and no *A. hydrophila* strains were isolated. The lack of *A. hydrophila* was probably due to the improvements made to the medium. However given the likely diversity within the species, it is possible that there are strains of *A. hydrophila* in the environment that will tolerate the medium and give false-positive results in the future. As a matter of good practice all positive SEC-PCR-EHA reactions should by investigated further to verify the basis of the positive finding. In this field trial the samples were collected blind, eliminating any possible sample bias. All

laboratory testing was conducted with no knowledge of the relationship between sample number and the disease status of the fish. Specificity of the test was further reinforced during the trial with apparently healthy fish from a non-diseased farm all clearly negative by SEC-PCR-EHA, and healthy looking fish from diseased sites giving prevalence values ranging from 0 to 18%, see Table 6.5.

Tenacibaculum maritimum.

The specificity of the test for *T. maritimum* was very high with no evidence of positive reactions occurring when testing fish from a non-diseased farm. Therefore, field specificity correlated with laboratory specificity, *i.e.* specificity is >99%. From an epidemiological perspective, non-infected fish will be correctly identified using the SEC-PCR-EHA system. This means that positive reactions occurring from a site with a history of *T. maritimum* are likely to be due to the target organism.

Samples collected from farms with a history of *T. maritimum* infection were first tested by SEC-PCR-EHA and any positive reactions were further tested by RT-PCR-EHA to give an indication of live bacterial carriage. The SEC-PCR-EHA results did not correlate with culture as more samples were positive by SEC-PCR-EHA than were positive by sub-culture from the SEC medium. The possible reasons for this difference have been discussed previously. However, there was a close correlation between the results achieved using the SEC-RT-PCR-EHA system and culture. The RT-PCR system estimated the live carriage rate to be 1%, while culture estimated the live carriage rate to be 0%. The observed proportion of agreement between the two tests was 98% (Cameron 2002), indicating that statistically the results given by either test are almost equivalent. Therefore the SEC-RT-PCR-EHA system proved to be very useful for approximating live carriage of the target organisms from a farms with a known history of disease.

Yersinia ruckeri.

The *Y. ruckeri* SEC-PCR-EHA system was validated by proving that the test specificity and sensitivity values obtained by laboratory testing are equivalent with field samples. Field samples did not adversely affect the test sensitivity as

determined by retrospective seeding. Therefore samples from fish that carry the bacterium to a level of 5 CFU per 0.1 g faeces (sensitivity of SEC, see Table 5.1) or more will give positive results using the system.

Specificity testing using Atlantic salmon from a farm with no history of disease was not possible due to the occurrence of the bacterium in all hatcheries in Tasmania. As a compromise, specificity testing was performed on rainbow trout from a hatchery that raises both Atlantic salmon and rainbow trout. Rainbow trout are resistant to infection with *Y. ruckeri* serotype 01b and it was expected that the samples would be negative. However, during the trial one EHA sample appeared to be positive, sub-culture from the corresponding SEC sample confirmed the presence of *Y. ruckeri*. No other positive EHA reactions occurred, including no 'ghost' bands by gel electrophoresis, indicating no history of infection. Therefore, field specificity correlated with laboratory specificity (>99%). This trial showed that non-infected fish will be correctly identified with the SEC-PCR-EHA system and that infected fish are also identified. The isolation of *Y. ruckeri* from rainbow trout faeces highlights the possibility that while these fish appear resistant to infection, they may actively carry the bacterium and serve as a reservoir.

Fish trials conducted from a farm with known yersiniosis gave the same kind of result as seen for *T. maritimum* where SEC-PCR-EHA suggested high carriage level but this could not be confirmed by culture. For instance the results of the first trial showed a 23% history of infection as determined by SEC-PCR-EHA, a 4% approximation of live carriage using RT-PCR and 0% carriage using sub-culture from the selective medium. If the results of the RT-PCR and culture from SEC are compared the observed proportion of agreement between the two tests was 'very good' (96%) (Cameron 2002), indicating that statistically the results given by either test are almost equivalent.

The second *Y. ruckeri* trial showed two different levels of carriage with the two populations of fish tested, *i.e.* vaccinated and unvaccinated fish. No live carriage was detected using the RT-PCR system with samples from fish that had been vaccinated and 4% live carriage was detected from fish that were unvaccinated. Therefore the RT-PCR system has identified that vaccination of fish not only prevents the development of fulminating disease, but may also reduce carriage rates of the bacterium. The 4% prevalence of *Y. ruckeri*

obtained in this trial is significantly less than the values obtained in previous testing. After testing this result was discussed with the farm manager who said that this finding complemented field observations, and that no *Y. ruckeri* had been isolated during routine microbiological health screening for some time.

Lactococcus garvieae.

Specificity testing undertaken for *L. garvieae* showed no false-positive reactions, indicating a test specificity of greater that 99%. Although there had been no recent history of disease in Tasmania, testing using SEC-PCR on fish from an outbreak in New South Wales gave some PCR positive, culture negative results, as seen for the other pathogens. *L. garvieae* was isolated from culture giving confidence in the capacity of the system to detect the bacterium. Although SEC-PCR-EHA could not be validated with positive samples from the field there is sufficient evidence based on seeded samples to indicate the validity of the system.

Comparison validation.

During the field trials positive SEC-PCR-EHA reactions were often negative by sub-culture from the corresponding SEC medium. Therefore the SEC-PCR-EHA was either more sensitive or less specific than culture making comparison validation between the two systems not valid. Given the >99% specificity achieved in the laboratory and the negative results achieved from specific pathogen free fish it is likely that the SEC-PCR-EHA is too sensitive to be compared with culture, probably due to the detection of DNA from dead cells.

The results of the SEC-(RT-)PCR-EHA and sub-culture were much more similar with 96% correlation or better in every case. However, the fact that there is even a slight difference between the two tests indicates that one of the tests is more sensitive or specific. As with the PCR test, due to the proven specificity it is likely that the RT-PCR test is more sensitive. Also as this RT-PCR system detects 16S rRNA (a molecule that is more stabile than mRNA) it is possible for RNA fragments from dead bacteria to cause positive results, possibly leading to a slight overestimation in the true level of live bacterial carriage.

In summary, field validation for each system was undertaken by performing SEC-(RT) PCR-EHA on samples collected from fish. Where possible, the specificity of the system has been confirmed with no positive reactions occurring from specific pathogen free fish. Sensitivity of the system with field samples was determined using retrospective seeding to be the same as that achieved with SEC media using purified nucleic acids. Therefore the system was either not affected by any chemical or biological inhibitors that were present in the samples, or all of the inhibitors were removed during the vacuum extraction procedure. Reliability of the system was determined by thoroughly testing the dynamics of the high-throughput system, trouble shooting where necessary, and by performing as many field trials as time would allow. A total of 10 fish trials were completed resulting in 1300 fish being tested. During these trials the SEC-PCR-EHA performed as expected with the EHA cut-off values proposed from laboratory testing holding true.

CHAPTER 7: General Discussion

This research resulted in the development of a sensitive technology to detect covert infections of *Aeromonas salmonicida*, *Tenacibaculum maritimum*, *Lactococcus garvieae* and *Yersinia ruckeri* in farmed salmonid fish. While conventional detection techniques such as culture, and newer methods such as direct PCR, reliably detect these organisms from fish that are overtly diseased, covertly infected fish are likely to be mis-diagnosed. The detection of covertly infected fish is essential for disease control, as through these fish the bacterium is actively spread to unaffected fish, increasing the percentage of carriers in the population or infecting previously unaffected stock. As covertly infected fish show no signs of disease, the status of the fish population is unknown and when stress occurs, fulminating disease in a large proportion of the stock can occur. The selective enrichment culture-(reverse transcriptase-) polymerase chain reaction-enzyme hybridization assay (SEC-(RT-)PCR-EHA) described here represents a practical means of detecting covert infections in fish. This was made possible by the incorporation of SEC media into the protocol, which enabled the use of a large sample size, inhibited the growth of non-target normal flora and encouraged the growth of the target pathogen to levels detectable by PCR. To further increase the likelihood of detecting covert infections the PCR, RT-PCR and EHA protocols were optimised for maximum sensitivity.

Development and validation of the SEC-(RT-)PCR-EHA was conducted following the protocol set out in Figure 7.1. This approach was taken to ensure that thorough validation of the system occurred at every step of the development process, that any problems would surface early and that the final system would be reliable and robust. Steps 1 to 3 of the protocol are based on the framework for validation studies of PCR-based bacterial detection techniques described by Hiney and Smith (1998b). However Hiney and Smith use comparison or predictive validation only for validation with field samples, while in this study the validation protocol was made more thorough by also validating the systems sensitivity and specificity from an epidemiological perspective.

7.1. Step 1: Application of technology

The system described here was developed to detect covertly infected farmed salmonid fish for disease surveillance and monitoring. The system is low-cost and streamlined for high-throughput sampling using micro-plate technology and one-tube PCR-EHA. In addition sampling procedures are simple, requiring little scientific expertise at the farm level and the testing procedure requires only a basic knowledge of PCR. This enables the technology to be easily adopted by even small veterinary laboratories. The system also allows for non-destructive sampling with three of the four pathogens.

The system uses two types of technology, PCR and RT-PCR. The results given by the two technologies are quite different, and the choice of technology is determined by the requirements of the investigation. As PCR detects both live bacteria and DNA that remains after cell death (Herman 1997), the detection of covert infection using PCR will demonstrate evidence of infection, past or present, making it an ideal tool for the surveillance of pathogens for purposes such as quarantine and certification of freedom from disease. However as ribosomal RNA gives a more accurate indication of the presence of live bacteria than DNA (Rosenthal and Landolo 1970) the RT-PCR technology is better applied to determining the proportion of active carriers in the population. Possible uses for covert infection prevalence values determined using RT-PCR include, the analysis of changes in carriage levels before and after vaccination and the determination of epidemiological causal and risk factors. When either technology is applied correctly detection of covert infection can allow better-informed management and decision making at the farm level.

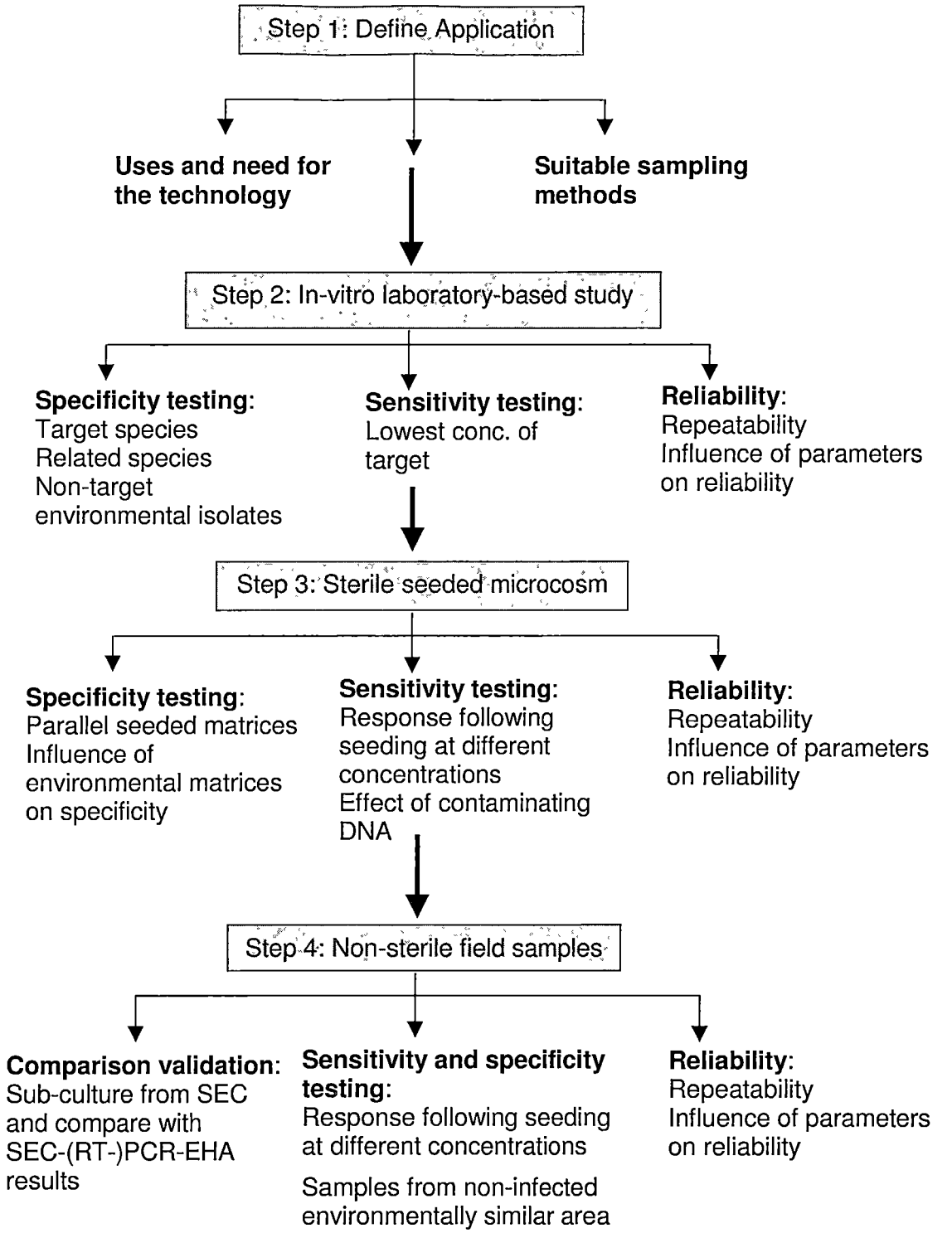


Figure 7.1: Validation protocol for SEC-(RT-)PCR-EHA.

7.2. Step 2: In-vitro laboratory-based study

The laboratory-based study began by optimising the existing PCR protocols and developing new 16S rRNA RT-PCR protocols to produce the best possible sensitivity without compromising specificity. Optimisation was achieved by varying the reagents of the PCR such as the *Taq* polymerase and by altering the PCR cycling conditions such as annealing temperatures and PCR extension times to suit each bacterium. A sensitivity of at least 4 fg of DNA or RNA was achieved for each species using purified nucleic acids. This sensitivity compares favourably with published sensitivity values for other bacteria such as, 1 pg for *Photobacterium damselae* ssp. *piscicida* (Osorio, *et al.* 1999) and 0.1 ng for *Aeromonas caviae* and *Aeromonas trota* (Khan and Cerniglia 1997). The specificity of the system was determined using phenotypically and genotypically similar bacteria to the target pathogen, the specificity matched that achieved by Carson (1998) using the same primer sets.

To help maintain the specificity of the system when testing field samples, internal hybridization technology was added to the system. This technology was incorporated into the system using NucleoLink™ micro-well enzyme hybridization tubes. Micro-well enzyme hybridization assays (EHA) allow for faster hybridization than blotting methods, easier high-throughput sample processing and provide a convenient format for visualising the results. As an added advantage, when using NucleoLink tubes, PCR and hybridization are performed in the same tube thus requiring that only one tube per sample from cDNA (for RT) to EHA is required, thereby decreasing the cost and time involved in sample transfer and decreasing the risk of cross-contamination between samples. Using NucleoLink tubes, the sensitivity and specificity values achieved using regular PCR tubes were maintained. Reliability of the assays was determined by repeating the assays many times throughout the optimisation and development stages.

Due to the sensitivity of PCR and the intended application of this technology, the prevention of false-positive reactions was seen as an essential requirement. To decrease the risk of positive reactions due to amplicon carry-over the amplicon inactivation agent IP-10 was incorporated into the PCR protocol. Existing protocols were readily adapted to include IP-10 and

inactivation was achieved using a standard UV transilluminator or a UV crosslinker. Assays were conducted to ensure that the IP1-0 did not adversely affect test specificity or sensitivity. Despite extensive investigation IP-10 could not be incorporated into the RT-PCR protocol due to a marked decrease in sensitivity. However, considering the minimal handling involved in the one-tube one-step RT-PCR process used in the RT-PCR-EHA technology, the risk of contamination is already greatly decreased and the absence of IP-10 was not considered a significant problem. Also, when the PCR technology is used for surveillance purposes, the frequency of use of the RT-PCR compared with the PCR system is such that RT-PCR posed much less threat of laboratory contamination than PCR.

7.3. Step 3: Sterile seeded microcosm

To determine the sensitivity of the complete system in the laboratory, SEC media was seeded with decimal dilutions of the target bacteria. Vacuum extraction followed by PCR and EHA were then performed. The detection limit achieved ranged from 4 to 16 CFU per sample, see Table 5.1. Competing DNA in the sample did not adversely affect sensitivity. The small reduction in sensitivity (as compared with 4 fg for the purified nucleic acids) was due to either slight inhibition in the SEC or imperfect nucleic acid extraction. For example, the *T. maritimum* PCR system required 4 CFU to produce a positive result. In this case, inhibitory agents in the SEC meant that 4 bacterial cells were required before growth in the SEC could reach a level detectable by PCR-EHA (as determined by MPN analysis). However, with the *L. garvieae* system 16 bacterial cells were required for a positive result. In this case the detection limit was due to inefficiencies in the DNA extraction technique. While the detection limit for *L. garvieae* is less sensitive than for the other bacteria, as the deficiency is at the extraction stage of the protocol there is unlikely to be any real reduction in sensitivity. This is because DNA extraction is performed after SEC incubation, at which time the density of the target pathogen in the SEC is likely to be much greater that 16 cells per 200 µl. The sensitivity values achieved using this protocol compare very favourably with other values achieved for SEC-PCR. For example, Giesendorf *et al.* (1992) required

100 CFU of *Campylobacter* spp. in 200 µl of medium for a positive result, while Lindqvist (1999) could detect 1-2.0 x 10^4 CFU *Shigella* spp. in 200 µl of enrichment broth.

The specificity of the system was not compromised when nucleic acids extracted from seeded SEC media were used for PCR. That is, no positive reactions occurred from samples that did not contain the target pathogen.

Reliability of the system was determined by preparing multiple sensitivity titrations and determining the effect of differing parameters on sensitivity. By performing these tests the optimum conditions for every step of the procedure were determined. Due to the close proximity of the samples to each other in a 96-well system every step of the high-throughput process was checked for cross-contamination. It important that the published methods are followed, especially the vacuum extraction procedure, to ensure the validity of the results.

7.4. Step 4: Non-sterile field samples

Validation of the SEC-PCR-EHA system using comparison validation was not successful due to the superior sensitivity of the SEC-PCR-EHA. However, validation of the SEC-(RT-)PCR-EHA system by comparison with culture was successful with 96% correlation or better in every case.

Ideally assessment of sensitivity and specificity from an epidemiological perspective should be conducted using a population of fish with known disease prevalence. However in this study the proportion of fish that actually carry the pathogen is not known, therefore sensitivity was assessed by comparing the detection limits achieved using the SEC-(RT-)PCR-EHA in the laboratory with the values achieved by retrospective seeding of field samples. Sensitivity of the system was not adversely affected by fish tissue or other biological variables contained in the field sample. PCR inhibitors are present in most environmental samples (Magnússon *et al.* 1994; Byers, *et al.* 2002), therefore it is likely that inhibitors were present in the SEC samples. Any PCR inhibitors were most likely removed during the vacuum extraction procedure. This was demonstrated with the RT-PCR system where performing 3 ethanol washes instead of 5 resulted in a reduction in test sensitivity. Due to the unknown disease status of the fish tested, the true sensitivity from an epidemiological perspective can not

be determined, however, with a detection level of between 4 and 16 CFU per sample it is likely that using this system all fish which carry the pathogen would test positive.

Specificity from an epidemiological perspective is the proportion of animals without the pathogen that test negative (Cameron 2002). Here, specificity of the system was assessed using fish from farms with no history of disease, as suggested by C. Baldock (AusVet Animal Health Services, Brisbane, pers. comm.). No false-positive EHA reactions occurred during the specificity testing, therefore the specificity of the system was determined to be >99%. Specificity can never be assumed to be 100%, as when testing new fish the possibility of false-positive reactions due to unknown organisms in the environment always exists.

7.5. PCR and RT-PCR detection strategies

PCR detects live bacteria and DNA from un-culturable and dead bacterial cells. The stability of bacterial DNA after cell death is unknown for these bacteria, but it is likely that positive PCR reactions could occur for many weeks after cell death (Herman 1997). Therefore the results of the SEC-PCR-EHA assay will not be useful for determining live bacterial carriage and may not be useful when monitoring changes in disease prevalence over a period of a few weeks or even months. In Chapter 6 the hypothesis that 'ghost' bands as seen by gel electrophoresis were due to un-culturable or dead bacterial cells was made. This hypothesis is supported by the inability to detect the organism by sub-culture from the SEC broths. Therefore for disease monitoring purposes it appears that the SEC-PCR-EHA system is too sensitive. For disease monitoring purposes carriage based on live bacteria is required and therefore an agreement between the SEC-(RT-)PCR-EHA results and culture from the SEC is desirable. This agreement is more probable using RT-PCR than PCR, as RT-PCR detects RNA which is more labile than DNA, breaking down much sooner after cell death (Rosenthal and Landolo 1970; Herman 1997), possibly giving a more accurate indication of live bacterial carriage.

Messenger RNA is the most labile form of RNA with a half-life of only a few minutes (Kushner 1996), and therefore gives a more accurate indication of live

infection than rRNA (Hellyer, *et al.* 1999; Yaron and Matthews 2002). However, the 16S rRNA gene primer sets developed for the four fish pathogens (Carson 1998) can not be used to detect mRNA. PCR based on mRNA would require a major change in primer design which can be complicated by the difficulty of finding a transcript that is constitutively expressed and species specific (McKillip, *et al.* 1998). A compromise would be to use RT-PCR using ribosomal RNA to approximate live bacterial carriage.

7.6. High-throughput and low-cost sampling

The intended application of the technology to detect covertly infected farmed salmonid fish for disease surveillance and monitoring was considered at all stages of development. This type of sampling requires a large number of fish to be sampled at the one time. Therefore, where possible the system uses a 96-well high-throughput format. A schematic diagram of the SEC-PCR-EHA system is given in Figure 6.1, Chapter 6. Following this diagram it can be seen that the system begins by adding a sample from a fish into a tube of SEC. Although it would allow for faster, more streamlined sampling this step is not performed in the 96-well format. This is because a 96-well format would only allow for a small sample size, and at this stage, as large a sample size as practical is required to ensure the detection of a pathogen that is present in very low numbers. The use of a 10 ml SEC volume allows for the addition of up to 500 mg of fish tissue without overloading the selective ability of the medium (Carson and Wilson 2001). When the target bacterium is present in this tissue it multiplies to a level where a small volume can be removed and the 96-well format begun. This form of high-throughput processing allows for the use of multichannel pipetting devices and a consistent format throughout the processing, greatly increasing throughput time and decreasing the risk of process error and contamination.

Another major consideration throughout the development of the SEC-PCR-EHA was the cost of sampling. Due to the high number of fish usually required for sampling, a cost-effective system was essential. For the PCR form of the test, the cost per sample is $2.50 while for the RT-PCR the cost is $3.30 per sample. This cost is considerably lower than would be incurred

if commercially available DNA extraction, PCR and enzyme hybridization kits were used.

Sampling costs could be lowered further if a system using pooled samples was used (Abel, *et al.* 1999), but the rationale for doing so would be dependent on the nature of the survey and the data required. Pooling samples may be an efficient way of lowering the cost of surveillance programs used to determine freedom from disease, or monitoring programs where obtaining an accurate prevalence value is not important.

7.7. Practical application for the SEC-(RT-)PCR-EHA technology

Prior to using the SEC-(RT-)PCR -EHA technology it is important to understand the purpose for which the testing is being undertaken. For determining small changes in the disease prevalence of a population or estimating the live carriage of a bacterium the RT-PCR system should be used. To determine the number of samples required to establish the prevalence of disease in a population, an estimate of the true prevalence must be made. Unfortunately the prevalence of disease is often hard to estimate, especially in populations where the prevalence has not been previously determined. In this case, to ensure enough fish are sampled, it is often good practice to estimate a disease prevalence to be 50%, as this results in the maximum samples size. The required precision of the results (the size of the confidence interval) also has an effect on the required sample size. If a high level of precision is required then more samples are needed. The amount of precision will vary depending on the application of the test, but as a rule as the prevalence gets smaller the required precision gets greater (Cameron 2002). Once these factors have been determined, the sample size for estimating the prevalence of disease is usually determined by using a computerised epidemiology program such as Win Episcope 2.0 (de Blas, *et al.* 2000).

When using the system to determine freedom from disease, or disease prevalence due to live and dead bacterial cells, the PCR system should be used. For determining freedom from disease the number of fish required for sampling depends on the test sensitivity and specificity. As discussed earlier, true field specificity and sensitivity values cannot be determined, as the true prevalence of

disease in the population is not known. However using specific pathogen free fish, the specificity of the system was determined to be >99%, and using retrospective seeding, laboratory detection levels of 4 to 16 CFU per sample were maintained. While determining the detection level of the system does not allow the sensitivity to be expressed as a percentage, knowing the exact sensitivity of the system is not as critical as specificity for determining sample size, this is clearly demonstrated in Table 7.1. (created using FreeCalc version 2, Cameron 2002).

Table 7.1: Effect of test sensitivity and specificity on sample size of determining freedom from disease.

Specificity	Sensitivity	Sample size
100	100	56
100	90	66
100	80	74
99	100	128
99	90	166
99	80	183
98	100	188
98	90	223
98	80	263

*Assumed population size 100 000, minimum expected prevalence 5%

In conclusion, this research has successfully developed a low-cost, high-throughput system for detecting covert infections of *Aeromonas salmonicida*, *Tenacibaculum maritimum*, *Lactococcus garvieae* and *Yersinia ruckeri* in farmed salmonid fish. The system has been thoroughly validated and has shown to be highly specific and far more sensitive than current detection methods for these pathogens. The system has already been used to assess the prevalence of *A. salmonicida* in Atlantic salmon after a disease outbreak, to

determine the risk of further outbreaks in the population and wider interest in the application of the technology for the detection of a range of bacteria has been forthcoming. It is anticipated that the system will continue to have a role in the detection of covert infections in the Tasmanian Aquaculture industry, thereby allowing improved disease control and reducing costs to the industry.

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APPENDIX A

Marine Sheih's Broth (Adapted from Song *et al.* 1988)

Peptone (Oxoid, Code L37)	5 g
Sodium acetate	0.01 g
Sodium pyruvate	0.1 g
Citric acid	0.01 g
Yeast extract	0.5 g
Aged sea water	900 ml
Distilled water	100 ml
pH	7.5 – 7.8

Autoclave for 15 minutes at 121 °C.

0.1M Saline-EDTA

Sodium chloride	8.75 g
EDTA disodium salt	37.2 g
Distilled water	1000 ml

Adjust pH to 8.0 with 2M NaOH.

Autoclave for 15 minutes at 121 °C.

50 mg ml^{-1} Proteinase K

Proteinase K*	50 mg
Distilled water	1 ml

*Proteinase K, *Tritirachium album*: Amresco (Product number: 0706)

20 mg ml^{-1} Lysozyme

Lysozyme*	40 mg
0.1M Saline-EDTA	2 ml

*Lysozyme, grade 1: Sigma (Product code: L-6876)

50xTAE buffer (use at 1x)

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5M EDTA	100 ml

Add ingredients and make up to 900 ml with water.
Dissolve the Tris and then make it up to 1 Litre.

10mM $MnCl_2$

Dilute 1M $MnCl_2$ stock (Sigma) to 10mM using 18 Mohm water.
Add DEPC to a final concentration of 0.2% to the solution, vortex well and let stand overnight.
Autoclave at 121 °C for 30 minutes.

10mM $CaCl_2$/ 0.9M Tris-HCl, pH 8.0

Prepare a stock of 100mM $CaCl_2$ using 18 Mohm water. Add DEPC to a final concentration of 0.2% to the solution, vortex well and let stand overnight. Autoclave at 121 °C for 30 minutes.

Add 10 µl of 100mM $CaCl_2$ to 90 µl of 1M Tris-HCl, pH 8.0 (AMRESCO, code E199).

20XSSC (stock concentration)

3M Sodium chloride

0.3M Sodium citrate

Prepared in RO (<2 µS) water and adjust pH to 7.0

10 × DIAPOPS buffer (100 ml)

TRIS-HCl (157.56 g M^{-1})	12.70 g
TRIS base (121.1 g M^{-1})	2.36 g
NaCl (58.44 g M^{-1})	8.76 g

The volume is adjusted to 99 ml with 18 Mohm water.

Autoclave at 121 °C for 30 minutes.

After autoclaving 1 ml of concentrated Tween 20 is added.

The pH must be checked and adjusted to 7.5 with 1 M NaOH.

Hybridization buffer

Hybridization buffer	Volume (µl) One strip	Volume (µl) 12 strips
20 x SSC	240	2880
100 x Denhardt's (Amresco)	40	480
10% Tween 20	8.0	96
RO water	503.2	6038.4
Total	791.2	9494.4

1 M Diethanolamine (pH 9.8) with 1 mM $MgCl_2$ (1000 ml)

Diethanolamine, MW = 105.14 g M^{-1}	95.6 ml
RO water (<2 µS)	800 ml

Adjust pH to 9.8 with 10 M NaOH or 10 M HCl

Then add 0.2033 g $MgCl_2$. 6 H_2O MW = 203.30 g M^{-1} and adjuste the volume to 1000 ml with RO water.

Diethanolamine buffer can be stored at 4ºC for at least 3 months.

APPENDIX B